Abundance, Diversity, and Dynamics of Viruses on Microorganisms in Activated Sludge Processes

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Abstract

We examined the abundance of viruses on microorganisms in activated sludge and the dynamics of their community structure. Direct counting with epifluorescence microscopy and pulsed-field gel electrophoresis (PFGE) were applied to 20 samples from 14 full-scale wastewater treatment plants (wwtps) treating municipal, industrial, or animal wastewater. Furthermore, to observe the dynamics of viral community structure over time, a laboratory-scale sequencing batch reactor was operated for 58 days. The concentrations of virus particles in the wwtps, as quantified by epifluorescence microscopy, ranged from 4.2×10^7 to 3.0×10^9 mL^{-1}. PFGE, improved by the introduction of a higher concentration of Tris–EDTA buffer in the DNA extraction step, was successfully used to profile DNA viruses in the activated sludge. Most of the samples from different wwtps commonly had bands in the 40–70 kb range. In the monitoring of viral DNA size distribution in the laboratory-scale reactor, some bands were observed stably throughout the experimental period, some emerged during the operation, and others disappeared. Rapid emergence and disappearance of two intense bands within 6 days was observed. Our data suggest that viruses—especially those associated with microorganisms—are abundant and show dynamic behavior in activated sludge.

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Introduction

Viruses are considered to be important components of natural microbial systems. For example, in water environments they are abundant and can affect bacterial communities through the lysis of bacterial cells or through horizontal gene transfer [6, 12, 13, 19, 35, 36, 48, 52]. It is suggested that 10–20% of the bacteria is lysed daily by viruses [43, 44], and viruses selectively lyse those microbes whose populations in the marine microbial community are large, thus influencing the richness of these populations [46, 47, 49].

Activated sludge processes have widely been employed to treat wastewaters from households and industrial activities. Activated sludge is essentially composed of different kinds of microorganisms ranging from bacteria to protozoa and metazoa. Also, the existence of viruses (such as bacteriophages) that infect microorganisms in activated sludge has been reported. Ewert and Paynter [11] reported an increase in viral numbers in activated sludge, as observed by electron microscopy. Khan *et al.* [22, 23] and Lee *et al.* [26, 27] isolated bacteria from activated sludge and applied them to plaque formation assays to look for bacteriophages: 30–60% of the bacterial isolates had related bacteriophages. However, little is known about the dynamics and abundance of the viruses associated with microorganisms in activated sludge.

There are several obstacles to viral community analysis. First (and most importantly), unlike the 16S rRNA genes targeted by universal primers in a majority of prokaryotic organisms, there is no universal target sequence for a wide range of viruses [38]. Until now, conserved DNA sequences for particular genes have been found in several viral groups; these include the DNA polymerase

gene in the Phycodnaviridae (algal viruses) [7, 8] and the *g20* capsid protein gene in cyanophages [14, 40]. However, there have been no reports on universal sequences that are possessed by all viruses. Second, as Amann *et al.* [1] have reported, fewer than one-tenth of the bacteria in the environment are known to be culturable; most of the bacteria that could be hosts for bacteriophages are thought to be unculturable, as would be their related bacteriophages [42]. Third, morphological and physiological characteristics are not strong keys for the identification or grouping of viruses [6, 37].

Pulsed-field gel electrophoresis (PFGE), which enables the separation of long-chain DNAs on the basis of their sizes, has been applied to analyses of viral community structure in the rumen [25, 45], in marine and freshwater environments [20, 21, 42, 50, 51], and in a solar saltern [39]. With PFGE analysis, viral DNA extracted from environmental samples can be profiled directly. This method gives an overall view of viral diversity without the various biases inherent in culture- and PCR-based assays [41]. Otawa *et al.* [34] reported the improvement of the PFGE method for application to the analysis of viral communities in activated sludge samples.

Direct counts made under electron or epifluorescence microscopy have shown viruses to be abundant in marine [4, 20, 33, 52], freshwater [17, 30], and soil [3, 48] environments. In contrast, there are few data on activated sludge environments, with the exception of the results of Ewert and Paynter [11].

Our goals were (1) to determine the density and community structures of viruses in various types of activated sludge processes and (2) to observe the dynamics of the viral community over time in a laboratory-scale activated sludge reactor by use of PFGE and direct counts under epifluorescence microscopy. To achieve our first goal, an improved PFGE method and direct counting by epifluorescence microscopy were applied to samples obtained from activated sludges at 14 full-scale wastewater treatment plants (wwtps). These wwtps treated municipal, industrial, or animal wastewater. For the second goal, we operated a laboratory-scale sequencing batch reactor (SBR) for 58 days and monitored viruses in it by the direct count method and by PFGE.

Materials and Methods

Activated Sludge Samples. Twenty activated sludge samples were collected from 14 full-scale wwtps in January, February, and March 2005. Some of the wwtps had plural wastewater treatment streams with different process configurations. The names of the samples, types of wastewater treated, process configurations, sampling months, and sampling sites are listed in Table 1. Thirteen samples were obtained from eight municipal wastewater treatment plants, five were from three animal wastewater treatment plants, and the remaining two were from two industrial wastewater treatment plants treating coke-oven wastewater. The process configurations employed included conventional activated sludge process, anaerobic–oxic (AO) system, anaerobic–anoxic–oxic (A_2O) process, SBR, and SBR with intermittent aeration. For each sample, 100 or 300 mL of activated sludge mixed liquor was collected from the aeration tank of the wastewater treatment plant for the analysis of

Table 1. Characteristics of the activated sludges investigated

Sample Name	Type of wastewater	Process configuration[a]	Sampling date (2005)	Prefecture
MIK-S	Municipal	Conventional	January	Tokyo
MIK-A	Municipal	AO	January	Tokyo
MIK-A2	Municipal	A_2O	January	Tokyo
KOS-S	Municipal	Conventional	January	Tokyo
KOS-A	Municipal	AO	January	Tokyo
NAK-S	Municipal	Conventional	January	Tokyo
NAK-A	Municipal	AO	January	Tokyo
NAK-A2	Municipal	A_2O	January	Tokyo
OCH-S	Municipal	Conventional	January	Tokyo
NAN-A	Municipal	AO	January	Tokyo
SUN-S	Municipal	Conventional	January	Tokyo
KAS-S	Municipal	Conventional	January	Tokyo
TSU-S	Municipal	Conventional	January	Chiba
SHI-S	Industrial (steel plant)	Conventional	February	Chiba
SHI2-S	Industrial (steel plant)	Conventional	February	Fukuoka
KAN-S	Animal (swine)	Conventional	February	Kanagawa
KAN-BI	Animal (dairy cattle and swine)	SBR (intermittent aeration)	February	Kanagawa
MIY-BI	Animal (dairy cattle)	SBR (intermittent aeration)	January	Miyagi
FUK-B	Animal (Swine)	SBR	March	Fukuoka
FUK2-B	Animal (Swine)	SBR	March	Fukuoka

[a]Conventional: conventional activated sludge system; AO: Anaerobic–oxic system; A_2O: Anaerobic–anoxic–oxic system, SBR: sequencing batch reactor; SBR (intermittent aeration): intermittent aeration-SBR.

viruses. The samples (except for samples SHI-S, SHI2-S, and MIY-BI) were immediately cooled to 4°C, carried to our laboratory, and subjected to further analyses within several hours after sampling. Samples SHI-S, SHI2-S, and MIY-BI were carried to our laboratory at 4°C and subjected to further analyses within 24 h. Viruses in all of the samples except SHI-S, KAN-BI, SHI2-S, FUK-B, and FUK2-B were analyzed by both the direct count method and the PFGE method. Samples SHI-S and KAN-BI were analyzed only by PFGE, and samples SHI2-S, FUK-B, and FUK2-B were analyzed only for viral count. Data on the concentrations of mixed-liquor suspended solids (MLSS) in those samples from wwtps treating municipal wastewater in Tokyo were provided by the Sewage Works Bureau, Tokyo Metropolitan Government.

Laboratory-Scale Activated Sludge Reactor. To investigate the dynamics of viral community structure, a laboratory-scale sequencing batch-activated sludge reactor with a working volume of 2.5 L was operated with synthetic wastewater as the influent for 58 days. One batch cycle was composed of 6 h, with the following phases: inflow phase (0.5 h), anaerobic phase (1.5 h), aerobic phase (2.5 h), sedimentation phase (1 h), and discharge phase (0.5 h). The wastewater was stirred with a mixer all the time, except during the sedimentation and discharge phases. Treated wastewater (1.25 L) was discharged in the discharge phase. Excess sludge was withdrawn at the end of the aerobic phase to maintain the sludge retention time at 9 days. Synthetic wastewater was supplied to the laboratory-scale reactor, which contained the following substrates per liter of tap water: yeast extract (27.2 mg), monosodium L-glutamate (H_2O) (305.9 mg), $CaCl_2$ $2H_2O$ (12.0 mg), $MgCl_2$ $6H_2O$ (123.5 mg), KCl (57.2 mg), NH_4Cl (24.0 mg), $(NH_4)_2SO_4$ (29.4 mg), K_2HPO_4 (24.5 mg), KH_2PO_4 (19.1 mg), and allyl thiourea (5.4 mg). Seed sludge for the reactor was derived from another laboratory-scale activated sludge process fed with L-glutamate as the main carbon source. Although not controlled, pH was always about 7.4 at the end of the anaerobic phase and about 8.2 at the end of the aerobic phase.

Viral monitoring by PFGE and by the direct count method was performed every 3 days at the end of an aerobic phase from day 13 to day 58 of reactor operation. Every 3 days, dissolved organic carbon (DOC) was measured by a total organic carbon analyzer (TOC-V; Shimadzu, Kyoto, Japan) and the concentration of MLSS was measured according to the Testing Methods of Sewage [18].

Elution of Viruses. For the direct count, an elution method by sonication was employed [9]. The sonication conditions were optimized on the basis of the count of eluted virus. Sonication of 10 W was applied to 10 mL of activated sludge mixed-liquor sample for 30 s with a sonicator (model 450 Sonifier, Branson Ultrasonics, Danbury, CT, USA). For PFGE, the protocol of Kim et al. [24] was used, i.e., $NaNO_3$ was added to the sample (30 mL) to a final concentration of 1 M, and the pH was adjusted to 7.0 by addition of 1 M NaOH. The sample was then mixed by a magnetic stirrer for 10 min to elute the viruses from the sludge sample.

Direct Count of Virus Particles. The eluted virus sample prepared by sonication as described above was filtered through a sterile 0.2-μm pore-size membrane filter (Advantec, Tokyo, Japan) to remove bacteria and larger organisms. Viruses were counted by epifluorescence microscopy using SYBR Green I (Molecular Probes, Eugene, OR, USA) for staining [33]. In brief, the filtrate was filtered through a 0.02-μm pore-size Al_2O_3 membrane filter (Anodisc, Whatman, Tokyo, Japan). The residue on the filter was stained with SYBR Green I (1/400 dilution in 0.02-μm-filtered sterile water) for 15 min, mounted in the presence of antifade solution (Molecular Probes), and observed under blue excitation with a microscope (model BX51, Olympus, Tokyo, Japan). The stained samples were either counted immediately after preparation or stored at −20°C within 1 week until counting. Before use, all working solutions (i.e., stain solution and ultrapure water) were filter-sterilized with a 0.02-μm pore-size Al_2O_3 Anodisc membrane filter and sterile syringes before use. The number of virus particles in each sample was counted from a single filter, and at least 200 particles per filter were counted from at least 10 randomly selected fields per filter. For the counting, LEICA QWin Pro Version 2.3 (Leica Microsystems, Wetzlar, Germany) was employed. In general, DN/RNase treatment is needed, as samples can contain free nucleic acids that can give positive errors. We conducted a preliminary experiment in which viral counting was done with and without DNase/RNase treatment. Because the results showed that the incorporation or omission of DNase/RNase treatment had no significant effect, we decided to omit the DNase/RNase treatment.

Pulsed-Field Gel Electrophoresis. PFGE analysis was basically conducted as described by Steward [41]. The eluted viral sample was centrifuged ($1000 \times g$, 10 min), then the supernatant was carefully transferred to another sterilized tube. Bacteria and higher organisms in the supernatant were removed by filtration through a sterile 0.2-μm pore-size filter (Advantec). The filtrate was treated with a mixture of DNase I (10 μg/mL or 32 U/mL; Funakoshi, Tokyo, Japan) and RNase A (10 μg/mL; Wako, Tokyo, Japan) for 0.5 h at 37°C to degrade any naked nucleic acids. After nuclease treatment, 25 mL of the sample was transferred to a polycarbonate ultracentrifuge tube

and centrifuged for 80 min at 20°C in a Type 70 Ti rotor with Optima-L ultracentrifuge (Beckman Coulter, Fullerton, CA, USA) at 30,000 × g. The pellet was resuspended in 100 μL of Tris–EDTA (TE) buffer, then purified with a Microcon-100 ultrafiltration unit (Millipore, Bedford, MA, USA) [41] and stored at 4°C. Here, instead of using 1× TE buffer, as has been employed by Steward [41], 100× TE buffer was used to suppress residual DNase activity in the activated sludge samples [34]. The DNA in the purified viral particles was released by warming at 60°C for 20 min, followed by cooling on ice. The sample containing the released DNA was carefully handled by pipetting as infrequently and as slowly as possible to avoid shearing the high-molecular-weight nucleic acids liberated from the viruses. The sample was mixed with 1/10 volume of 10× loading buffer (Takara, Tokyo, Japan) and then subjected to PFGE.

PFGE was performed with a contour-clamped homogeneous electric field DR-III Cell (Bio-Rad Laboratories, Hercules, CA, USA) under the following conditions: 1% SeaKem Gold agarose (FMC BioProducts, Rockland, ME, USA); 0.5× TBE gel buffer (45 mM Tris-borate and 0.5 mM EDTA, pH 8.0); 0.5× TBE tank buffer; 1- to 10-s pulse ramp; 6 V/cm; 14°C; and 18 h (run time). The molecular weight marker run on the gels was a mixture of Molecular Weight Marker for DNA 0.1–200 kb from Lambda phage (Sigma-Aldrich, St. Louis, MO, USA) or MidRange II PFG marker (New England Biolabs, Ipswich, MA, USA). After electrophoresis, the gels were stained for 30 min in 1× SYBR Green I (FMC) in accordance with the manufacturer's instructions, and then digitally scanned for fluorescence by using a fluorescent image scanner, FluorImager (Molecular Dynamics, Sunnyvale, CA, USA).

Another experiment was conducted to observe the effect of residual DNase activity from an activated sludge sample, as reported by Otawa *et al.* [34]. T4 phage (Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan) was added to an activated sludge sample taken from the MIK wwtp in November 2005, and the sample then underwent viral analysis by PFGE by the method as described above.

DGGE/T-RFLP Analysis of Bacterial Communities. To examine their bacterial community structures, activated sludge samples were subjected to denaturing-gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP). A FastDNA SPIN kit (Qbiogene, Carlsbad, CA, USA) was used to extract DNA from activated sludge samples. DGGE analysis was conducted via the method of Muyzer *et al.* [32] with the primers 341f-GC and 518r. T-RFLP analysis was conducted as described by Moeseneder *et al.* [31] with the primers 27f-FAM and 1492r,

and 20 U of the restriction enzyme *Hha*I (Toyobo, Osaka, Japan). All of these four primers were complementary to sequences commonly conserved in most eubacteria.

Results

Viruses in Full-Scale wwtps. Virus concentrations in 18 activated sludge samples from the full-scale wwtps were analyzed by the direct count method with epifluorescence microscopy aided by SYBR Green I stain, and the results are listed in Table 2. The virus particle concentrations ranged from 4.2×10^7 mL^{-1} (SHI2-S) to 3.0×10^9 mL^{-1} (KAN-S), and the average was 1.1×10^9 mL^{-1}. The concentrations of virus particles per gram (dry) of MLSS ranged from 3.7×10^{11} to 8.3×10^{11}.

Distributions of viral DNA sizes were analyzed by PFGE (Fig. 1). For all samples, the sizes of viral DNA ranged from 20 to >200 kb, with most of them in the range of 40–70 kb. The larger genomes (>200 kb) observed in most samples (MIK-S, MIK-A2, NAK-S, NAK-A, NAK-A2, SUN-S, KAS-S, TSU-S, SHI-S, OCHI-S, KAN-S, and KAN-BI) were not well resolved under the conditions used, as can be seen from the size-marker lane. The resolution of the profile was not good in many

Table 2. Concentrations of microorganism-associated viruses in activated sludges from wastewater treatment plants

Sample name	MLSS (mg L^{-1})	Concentrations of viruses[a] (10^8 mL^{-1})	Concentrations of viruses[b] (10^{11} g^{-1} [dry])
MIK-S	1320	10.9 ± 1.1	8.3 ± 0.8
MIK-A	1390	10.7 ± 1.0	7.7 ± 0.7
MIK-A2	1980	7.8 ± 0.3	3.9 ± 0.2
KOS-S	1770	8.0 ± 0.7	4.5 ± 0.4
KOS-A	1370	8.1 ± 0.9	5.9 ± 0.6
NAK-S	1290	10.0 ± 1.0	7.8 ± 0.8
NAK-A	1680	9.7 ± 1.0	5.8 ± 0.6
NAK-A2	1550	5.7 ± 0.9	3.7 ± 0.6
OCH-S	2160	9.8 ± 1.1	4.5 ± 0.5
NAN-A	1430	7.4 ± 1.3	5.2 ± 0.9
SUN-S	1555	9.8 ± 1.8	6.3 ± 1.2
KAS-S	1620	13.5 ± 1.5	8.3 ± 0.9
TSU-S	N.A.[c]	24.5 ± 2.4	–
SHI2-S	N.A.	0.42 ± 0.1	–
KAN-S	N.A.	30.1 ± 11.3	–
MIY-BI	N.A.	3.0 ± 0.6	–
FUK-B	N.A.	13.9 ± 2.0	–
FUK2-B	N.A.	12.7 ± 4.0	–
SBR (day 34)	1825	22 ± 10.2	12.2 ± 5.6
SBR (day 40)	1375	6.3 ± 3.1	4.7 ± 2.2
SBR (day 43)	1875	16 ± 2.6	8.6 ± 1.4
SBR (day 46)	1835	58 ± 16	31.7 ± 8.9

[a]Concentrations of viruses were determined by a SYBR Green Q-based direct microscopic counting. Each value represents the mean count from at least 10 selected fields (standard deviations are shown with ±).
[b]Values were calculated from the numbers of viruses per milliliter and the MLSS data.
[c]Not analyzed.
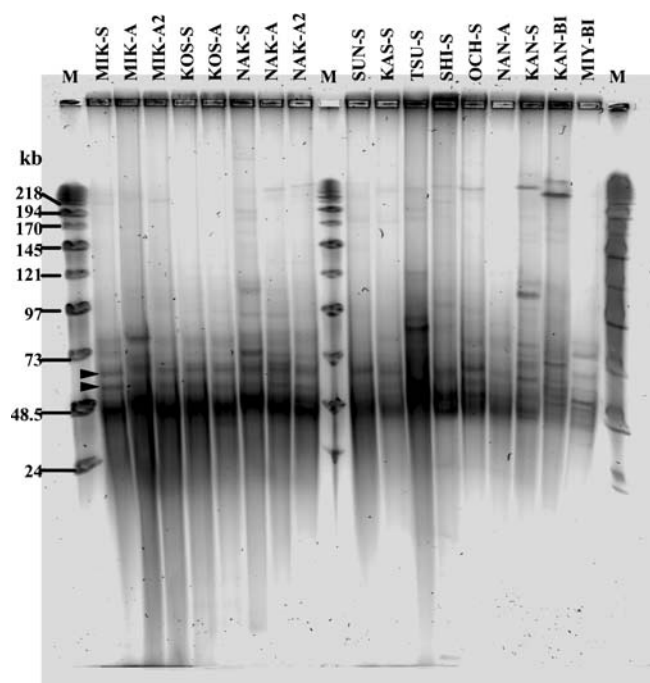
Figure 1. Pulsed-field gel electrophoresis: images of fluorescently stained viral DNA. Seventeen samples were collected from 11 of the wastewater treatment plants shown in Table 1. Lane M shows molecular size markers.

of the samples, especially at about 48 kb. However, addition of the T4 phage to an activated sludge sample made a distinct band (Fig. 2).

The PFGE profiles of most of the municipal and industrial wwtps tested (MIK-S, MIK-A, MIK-A2, KOS-S,
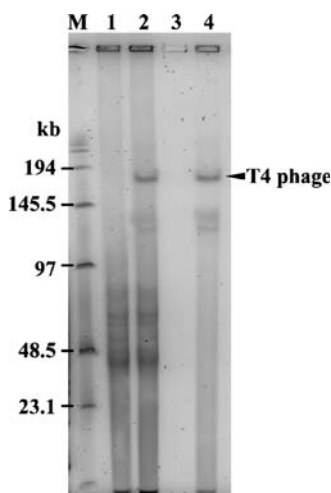
Figure 2. Pulsed-field gel electrophoresis profiles of a test of T4 phage addition to determine the effects of residual DNase activity in activated samples from wastewater treatment plants (wwtps). M: molecular size markers; 1: DNA extracted from concentrated total viruses in activated sludge from MIKwwtp; 2: mixture of extracted DNA (same as in lane 1) and T4 phage (168 kb); 3: same as in lane 1, but autoclaved before T4 phage addition; lane 4: same as in lane 2, but autoclaved before T4 phage addition.

KOS-A, NAK-S, NAK-A, NAK-A2, SUN-S, KAS-S, SHI-S, OCH-S, and NAK-S) had the most intensive bands at about 50 kb, although they were smeared (Fig. 1). Also, most of these samples commonly had particular bands with sizes of about 55 and 65 kb (indicated by arrows in Fig. 1). TSU-S, a municipal wwtp, had a different pattern from the other municipal wwtps, as it had bands with larger sizes. The animal wwtps tested (KAN-S, KAN-BI, MIY-BI) had less smearing and showed several clear bands between 40 and 70 kb; they thus had band patterns different from those of the municipal and industrial wwtps. KAN-S and KAN-BI were characterized by intensive bands at about 200 kb.
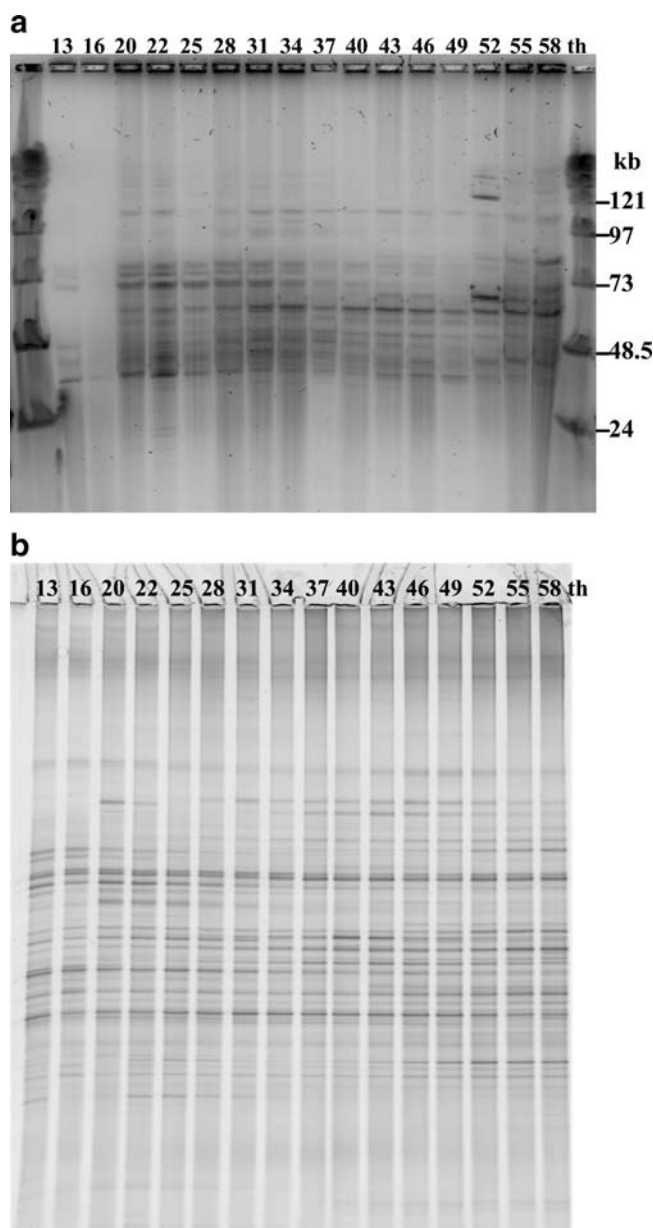
Viral Dynamics in the Laboratory-Scale Reactor. To monitor the dynamics of viral community structure over a time, we operated a laboratory-scale reactor for about 2 months and monitored viruses in it by direct counting methods and PFGE. The behaviors of MLSS and DOC in the reactor are shown in Fig. 3. The concentrations of MLSS fluctuated in the range of 780–2070 mg/L. Right after the start of the monitoring, a large part of activated sludge was lost in an accident, so during days 16 to 20 additional seed sludge was inoculated into the reactor. The removal efficiency of DOC was about 75% throughout the reactor operation, except on day 13.

The concentrations of virus particles in the activated sludge mixed liquor were 2.2×10^9 mL^{-1} (day 34), 6.3×10^8 mL^{-1} (day 40), 1.6×10^9 mL^{-1} (day 43), and 5.8×10^9 mL^{-1} (day 46) (Table 2). Viral particle concentrations per gram (dry) of MLSS were calculated as 1.2×10^{12}, 4.7×10^{11}, 8.6×10^{11}, and 3.2×10^{12} g^{-1} on days 34, 40, 43, and 46, respectively.

The dynamics of viral community structure in the laboratory-scale reactor were investigated by PFGE
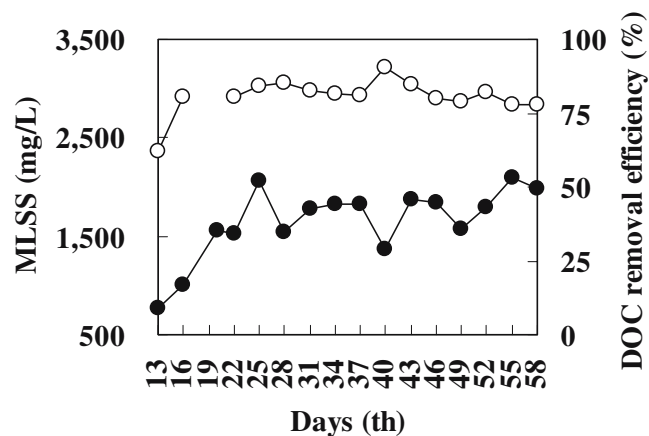
Figure 3. Changes in mixed liquor suspended solids (MLSS) concentration and dissolved organic carbon (DOC) removal efficiency in the laboratory-scale reactor over 45 days. MLSS (mg/L) is shown by solid circles and DOC removal efficiency (%) by open circles.

Figure 4. (a) Pulsed-field gel electrophoresis: images of fluorescently stained viral DNA. During the monitoring period (45 days) an activated sludge mixed liquor sample was collected every 3 days from the laboratory-scale reactor at the end of the aerobic phase and analyzed. Numbers of days after the start of reactor operation are shown at the top of the image. (b) Denaturing-gradient gel electrophoresis: images of 16S rRNA amplicons from bacterial DNA every 3 days in the laboratory-scale reactor. Numbers of days after the start of reactor operation are shown at the top of the image.

(Fig. 4a). The sizes of viral DNAs in all samples ranged from 40 to 140 kb, with most in the range of 40–70 kb. These patterns were different from those in full-scale wwtps. Decline of three or more dominant viruses was observed after day 37 (70–100 kb). On day 52, two dense

bands that had never been observed before (approximately 70 and 120 kb) emerged, but both of them disappeared quickly in the subsequent samples. The behavior of the bacterial community was monitored by DGGE (Fig. 4b) and T-RFLP (data not shown), but we could find no clear relationship between the bacterial community change monitored by these methods and the viral community change monitored by PFGE.

Discussion

We analyzed viruses in activated sludge by the direct count method and by PFGE. These methods themselves cannot distinguish between the nucleic acids of viruses and other nucleic acids. Treatment with DNase and RNase is thought to be effective in eliminating the error caused by naked nucleic acid molecules that might be present in samples. We therefore introduced DNase/RNase treatment in our pretreatment for PFGE analyses. We also checked the effect of DNase/RNase treatment on the direct count numbers, and because we found no significant difference—and to simplify the analytical procedure—we omitted DNase/RNase treatment for direct count measurement. Although there remains the question of whether the nucleic acids detected by PFGE and direct count were in fact from virus particles, the particles that we detected had characteristics in common with viruses: they were mostly protected by structures such as capsids from nuclease activity. Although it may be controversial to consider these particles as viruses, we would like to presume that they were [20, 34].

Viruses in activated sludge have two possible origins: they can be contained in influent wastewater or produced in the activated sludge. Generally, *Escherichia coli* phages (coliphages) are abundant in influent wastewater, although in 1965 Dias and Bhat [10] indicated that within 2 h of aeration of activated sludge, the coliphage population declined 10-fold from 2460 to 230 plaque-forming units (PFU)/mL. In 2005, Arraj *et al.* [2] also reported that the population of the RNA coliphage MS2 was reduced by 3 orders of magnitude in activated sludge. Previous studies have reported that coliphages are not functional in activated sludge [5, 11, 16, 28]. On the other hand, total virus particle concentrations have been shown to increase by 3.5 to 18.3 times in the course of wastewater treatment by activated sludge [11], suggesting that the presence of hosts in the activated sludge can promote viral reproduction [16]. Thus, the virus particles that we observed were mainly those produced through treatment. Indeed, we observed numbers of virus particles in our laboratory-scale activated sludge, which had been fed with synthetic wastewater that was essentially free from viruses.

We considered that the hosts of the viruses were mostly bacteria in the activated sludge, because the

majority of microscopically observed cells in activated sludge are indeed bacteria [29]. Nevertheless, it is also possible that the viruses were hosted by higher organisms such as protozoa or fungi in the activated sludge.

In the first part of this study, we determined the concentrations of viruses in full-scale wwtps treating municipal, industrial, or animal wastewater. Our results revealed that the viral particle concentrations in activated sludges in full-scale wwtps and in the laboratory-scale reactor were of the order of 10^8 to 10^9 mL^{-1}. Ewert and Paynter [11], using transmission electron microscopy, reported the viral concentrations in raw wastewater and activated sludge treated wastewater to be 3.0×10^6 to 9.5×10^7 mL^{-1}. One of the possible reasons why we obtained higher values is that our procedure included a step of elution of the viruses from the activated sludge, whereas that of Ewert and Paynter [11] did not. Another possible reason is the difference in counting methods. Whereas Ewert and Paynter [11] employed transmission electron microscopy, we employed epifluorescence microscopy with SYBR Green I. Enumeration by transmission electron microscopy have been reported to underestimate viral abundance because of technical problems such as uneven collection, uneven staining, washing off of viruses, and lack of recognition of atypical viruses [46].

The concentration of virus particles in activated sludge was found to be higher than in any other environment ever studied [3, 4, 9, 20, 30, 48, 52]. Total viral concentrations in marine environments range between approximately 10^4 and 10^8 mL^{-1} [52], and the highest concentration of viruses observed so far is 1×10^9 mL^{-1} [20]. In marine environments, viruses have been proposed to regulate the population densities of their host bacteria or the diversity of bacterial communities [12, 13, 43, 44]. Given the results of these studies, the high viral concentrations in activated sludge indicate that viruses have high activity not only in marine environments but also in activated sludge environments.

As activated sludge contains concentrations of microorganisms as high as a couple of grams per liter, the concentration of the virus relative to that of its potential host is also of interest as an indicator of viral activity in the microbial community. The number of virus particles in a given weight of MLSS is an indicator of relative viral concentration, as MLSS roughly corresponds to dry biomass. Viral concentration in the MLSS was calculated as 10^{11} to 10^{12} g^{-1}.

We introduced the PFGE method for the analysis of viral community structure in activated sludge. The PFGE method has been applied to viruses from marine and freshwater environments [20, 21, 42, 50, 51], the rumen [25, 45], and a solar saltern [39]. These studies have revealed that viruses can be separated well by PFGE according to their DNA sizes, enabling the comparison of viral community structures in different environments and at different times. Furthermore, with PFGE analysis, viral DNA extracted from environmental samples can be directly profiled without distortion of the virus community [42]. However, a disadvantage of PFGE is that it is difficult to distinguish bacteriophages from viruses that infect higher organisms such as protozoa, metazoa, and fungi. SYBR Green I, the dye that we employed in the PFGE analysis, is thought to be mainly applicable to double-stranded DNA and, therefore, the PFGE bands that we obtained essentially represent the genomes from DNA viruses. If SYBR Gold, a dye that is applicable to both double- and single-stranded DNA and RNA, were employed, single-stranded DNA viruses and RNA viruses might be detectable.

Otawa et al. [34] reported that the PFGE method described by Steward [41] cannot immediately be applied to activated sludge samples. They experimented with adding three known phages to activated sludge samples after the samples had been treated with DNase/RNase. As bands for these added phages were not observed, they concluded that the viral DNA was degraded in the liberation step of viral DNA. They further reported that DNA degradation can be avoided by the use of TE buffer at a higher concentration in the liberation step. We employed the method modified by Otawa et al. [34] to avoid the adverse effects of DNase on our activated sludge samples. Thanks to the modification, when T4 phage was added to an activated sludge sample, degradation of viral DNA by residual DNase activity was successfully avoided (Fig. 2).

The PFGE analysis of the 17 samples from the full-scale wwtps showed that all of the samples had bands ranging mainly from 40 to 70 kb. Although the band patterns from municipal and industrial wwtps (except TSU-S) were similar, those from animal wastewater were markedly different. Smear band patterns were observed at about 48 kb in many of the samples (except KAN-BI and MIY-BI), despite the introduction of 100× TE buffer. However, DNase activity was successfully suppressed (Fig. 2), and we therefore consider that the smear did not result from partial degradation of DNA by residual DNase activity. This smear appears to have been caused by the presence of viruses of similar size and concentration. In fact, the genome distribution of viruses from a variety of marine systems averages approximately 50 kb [42, 46]. Generally, the conditions of DNA extraction can also affect the smearing of viral DNA [41], but this effect should have been small, because DNA from the T4 phage was not smeared (Fig. 2).

It is interesting to examine the size distribution of viruses from activated sludge in comparison with those from other environments. In seawater, most viral DNAs are in the range of 23–97 kb [42, 50]. Viral DNA size distributions in a solar saltern (10–533 kb) [39] and the

rumen (10–500 kb) [45], investigated by PFGE, ranged more broadly than ours. Thus, the viral DNA size distribution in wwtps appeared to be in the same range as that in seawater and in a narrower range than that in a solar saltern or the rumen.

It is notable that similar PFGE band patterns were observed among 13 samples from seven municipal wwtps. Moreover, two PFGE bands (at 55 and 65 kb) were commonly observed among municipal wwtps, indicating the existence of identical or similar viral species (see Fig. 1). Although all of these wwtps were treating municipal wastewater, the sources of the municipal wastewater were different, and the operational conditions of the activated sludge processes were different. We cannot explain why similar band patterns were observed in these samples from municipal wwtps, but these similarities in viral community structure may be related to similarities in microbial community structure. If these common bands represent identical viruses that exist among different plants, then these viruses may have universal, yet unknown, roles in activated sludge microbial systems. The presence of common viruses among different wwtps is interesting in ecological terms, and to confirm this, it may be worth applying other molecular methods such as hybridization [51].

The PFGE method was also applied to the monitoring of viruses in the laboratory-scale activated sludge reactor. The laboratory-scale reactor had no influx of viruses, because synthetic wastewater was used. Therefore, we consider that the virus dynamics came from virus–host interaction inside the activated sludge itself. Different temporal behaviors of the viruses were observed: some were constant throughout the operation, some were observed only for short periods, and some declined after day 37 (Fig. 4a). On day 52, a totally different band pattern was observed: the observed emergence and disappearance of viruses was, in general, gradual with the exception of those bands that appeared suddenly at about 70 and 120 kb on day 52.

The existence of stably observed bands and the slowness of the emergence and disappearance of the bands indicate that there was a stable relationship between the viruses and their hosts, whereby both virus and host coexisted and the viruses did not lyse their hosts quickly. As causes of this stable relationship between viruses and hosts, we can suggest lysogenization, acquisition of resistance, and the existence of the physicochemical barrier provided by the floc structure of the activated sludge.

The emergence of the two bands observed on day 52 in the PFGE profile may have been caused by the bursting of host bacteria by their phages. However, no clear change in the host bacterial community was observed by our DGGE or T-RFLP analyses of the bacterial community (Fig. 4b). There are some possible causes for this result: (1) the burst-like observation might have represented the action of viruses lytic to protozoa, metazoan, or fungi; or (2) the population of the hosts that gave rise to the burst-like band might have been under the detection limit by DGGE or T-RFLP. We estimated the numbers of lysed bacteria that could have been involved in the burst to be $1.6–3.4 \times 10^7$ cells in the area of the 120-kb band and $4.3–8.9 \times 10^7$ cells in the area of the 70-kb band. We used the following data in our estimations: the genome sizes of the bands (120 or 70 kb), the general weight per base (5.128×10^{-13} ng), the DNA concentration of the marker used (50 ng/μL), the fluorescence intensity of each burst-like band relative to that of a 48.5-kb marker band, and the average number of virus particles produced per lysed bacterium (burst size) (24 or 50, respectively) [52]. The lysed bacteria were estimated to account for 0.13% (120-kb band) and 0.35% (70-kb band) of the total bacteria in the activated sludge when a value of 1.5×10^9 was applied as the general number of total bacterial cells per ml [15]. These percentages are low, and it might be difficult to detect this scale of bacterial change.

Conclusion

We applied the direct count and PFGE methods to the analysis of viral community structure in full-scale and laboratory-scale activated sludge processes. The following outcomes were obtained:

(1) Viral concentrations in activated sludges from both the full-scale wwtps and the laboratory-scale reactor were about 10^8 to 10^9 mL^{-1}.
(2) The sizes of viral DNAs from full-scale wwtps ranged from 40 to >200 kb, with 40 to 70 kb the most frequent.
(3) Similar PFGE band patterns were observed among municipal wwtps, indicating the existence of identical or similar viral species common to activated sludges used in the treatment of municipal wastewaters.
(4) Different temporal behaviors of viruses were observed in the laboratory-scale reactor. Some were stably observed throughout the operation and some were observed only for short periods. Two bands emerged and disappeared within a very short time during the monitoring of the laboratory-scale reactor.

As a whole, the concentrations of viruses in the activated sludges were as high as, or higher than, those in other natural environments. In addition, a clear temporal change in the virus community structure was apparent. These findings suggest that viruses are part of the microbial systems in activated sludges, although their roles have not yet been clarified. We think that further careful investigation of the effects of viruses on their hosts in activated sludges is necessary.

Acknowledgments

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