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Population Dynamics of Phage-Host System of *Microlunatus phosphovorus* Indigenous in Activated Sludge

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Abstract Monitoring of the phage-host system of *Microlunatus phosphovorus* indigenous in activated sludge was attempted. A laboratory-scale activated sludge process was operated for 5 weeks with synthetic wastewater. The phage-host system population in the process was monitored by plaque assay and FISH methods at every 3 days. During the process operation, the phage-host system populations were more or less steady, except for 1 week in the middle of the operation. In that period, initially *M. phosphovorus* decreased significantly and its lytic bacteriophages increased, and then *M. phosphovorus* increased back to its original level while its lytic bacteriophages decreased. This observation suggests that lytic bacteriophages should be considered as one of the biological factors affecting the bacterial population dynamics in activated sludge processes.

Keywords: Phage-host system, activated sludge, indigenous microbial population

Bacteriophages have been reported to exist in activated sludge processes [3, 5, 7, 10, 16]. Even though it is well known that bacteriophages can lyse their host bacteria, their effect on the host bacterial population in activated sludge is not properly understood yet. The effect of bacteriophages on the bacterial population, by viral-lysis activity, in a marine environment has been reviewed and it was suggested to be approximately between 15% to 20% of the bacterioplankton population across a wide range of marine environments [19]. Here, the effect of bacteriophages on the microbial population in activated sludge is of interest. Lee *et al.* [11] isolated two bacteriophages specific to *Microlunatus phosphovorus* from activated sludge in which both were native. Lee *et al.* [12] reported that the artificial boost of the bacterial host *M. phosphovorus* did not significantly affect its bacteriophages population in activated sludge, but there was a strong relationship between the population fluctuations of the bacterial host and its lytic bacteriophages in a reverse tendency. Here is a question about the possibility: is the host bacterial population "indigenous" in activated sludge negatively affected by the lysis activity of bacteriophages? If so, lytic bacteriophages should be considered as one of the biological factors affecting the bacterial population dynamics in activated sludge. Recently, some microbial ecologists have come to presume that the lysis activity of bacteriophages might change the bacterial community and structure in activated sludge processes [17]. However, very little is known about the phage-host ecology in an activated sludge process. Therefore, it is timely work to look into how lytic bacteriophages affect the host bacterial population in activated sludge. For that, in the present study, a laboratory-scale activated sludge process was operated and *M. phosphovorus* was targeted as the bacterial host indigenous in the activated sludge [14]. During the process operation, the populations of *M. phosphovorus* and its lytic bacteriophages were monitored.

A laboratory-scale activated sludge process was operated for 5 weeks under the sequencing anaerobic and aerobic conditions at 20°C. One cycle of the sequence comprised 30 min of feeding phase, 90 min of anaerobic phase, 150 min of aerobic phase, 60 min of settling phase, and 30 min of discharge phase. In each cycle, 1,250 ml of influent synthetic wastewater was supplied in the feeding phase, and 1,250 ml of treated water was discharged in the discharge phase. The composition of the synthetic wastewater was 314 mg sodium glutamate·H_2O, 28 mg yeast extract, 12.3 mg $CaCl_2 \cdot 2H_2O$, 127 mg $MgCl_2 \cdot 6H_2O$, 58.8 mg KCl, 24.6 mg NH_4Cl, 30.2 mg $(NH_4)_2SO_4$, 18.2 mg K_2HPO_4, 19.6 mg KH_2PO_4, and 5.6 mg allylthiourea (ATU) per liter. The working volume of the sequencing batch reactor (SBR) was 2,500 ml. The pH in the SBR was between 7.2 and 8.2. The seeding sludge was from an urban wastewater

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treatment plant in Tokyo, Japan. The sludge retention time (SRT) was maintained at 9 days. Before the seeding, the sludge had been operated in another SBR fed with similar synthetic wastewater for approximately 6 months. The synthetic wastewaters were sterilized before feeding. *Microlunatus phosphovorus* JCM9379, which was originally isolated from activated sludge [14], obtained from Japan Collection of Microorganisms (JCM), was incubated at 25°C in the medium equivalent to the 36-times-concentrated synthetic wastewater. This bacterial strain and medium were used for quantification of *M. phosphovorus*-lytic bacteriophages in the activated sludge.

M. phosphovorus-lytic bacteriophages in the activated sludge were counted by using the plaque assay method [1, 8]. Activated sludge mixed liquor sampled in the end of the aerobic phase of the SBR was centrifuged, and the supernatant was filtered through a 0.20-μm-pore-size membrane filter (Millipore). The filtrate of 200 μl was mixed with 3.5 ml of the soft agar medium (0.7% agarose) containing 1 ml of exponentially growing *M. phosphovorus* JCM9379. The mixture was poured into plates over the preset agar medium (1.5% agarose). The plates were incubated upside down at 25°C for 10 days to observe plaques on the lawn of *M. phosphovorus* JCM9379. After incubation, plaques were counted, and then the concentration of *M. phosphovorus*-lytic bacteriophages was calculated as plaque forming unit (PFU). This quantification was conducted at every 3 days in duplicate.

The population of *M. phosphovorus* in the activated sludge was quantified by the fluorescence *in situ* hybridization (FISH) method, at every 3 days. The 1-ml activated sludge mixed liquor sampled from the end of the aerobic phase was centrifuged and the sludge pellet was immediately stored at −80°C. Fixation and *in situ* hybridization were performed as described by Amann *et al.* [2] and Kawaharasaki *et al.* [6] with minor modification. After thawing the sludge pellet in 1 ml of phosphate-buffered saline (PBS; 130 mM sodium chloride, 10 mM sodium phosphate buffer, pH 7.2), it was sonicated at approximately 8.5 W for 2 min. The sample was fixed overnight with 99.5% ethanol and then the ethanol was removed by centrifugation. The resuspended sample in 1 ml of 10 mM phosphate buffer (pH 6.5) was treated with lysozyme (0.1% lysozyme, 30 min at 37°C) before the addition of the same volume of 99.5% ethanol. The sample was immobilized and dehydrated for *in situ* hybridization. Oligonucleotide probe MP2 (5'-GAGCAAGCTCTTCTGAACCG-3') [6] labeled with Cy3 was obtained from Proligo for the specific detection of *M. phosphovorus*. Hybridization was carried out at 46°C for 2 h at the formamide concentration of 10%. The washing of the unhybridized probes and 4',6-diamidino-2-phenylindole (DAPI) staining (2 μg DAPI/ml) were performed as described by Amann *et al.* [2], Kim *et al.* [9], and Lee *et al.* [13]. Microscopy was performed

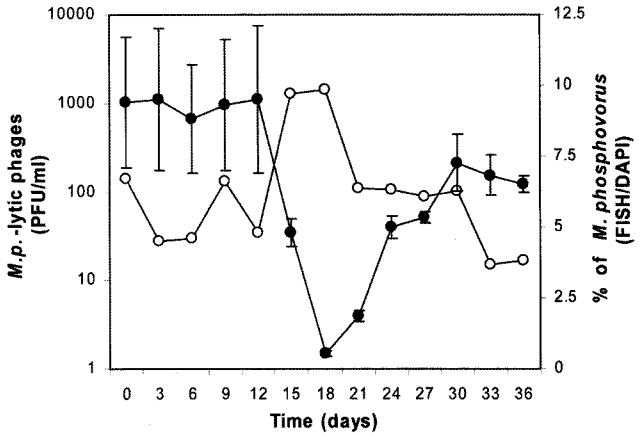

Fig. 1. Population dynamics of *M. phosphovorus* and its lytic bacteriophages during the process operation.
(●) *M. phosphovorus* (SD bars are shown on the figure), (○) *M. phosphovorus*-lytic bacteriophages (SD ranged from ±3 to ±27 PFU/ml, cannot be shown on the figure because of the value scale).

with an epifluorescence microscope Olympus BX51 (Olympus Optical) equipped with a CCD camera (DP-50, Olympus Optical) at ×1,000 final magnification. The quantification of *M. phosphovorus* was performed as the area percentage of the FISH-positive cells against the area of DAPI-positive cells from more than five microphotographs of each sample using the Leica Qwin software version 2.3a (Leica Microsystems Imaging Solutions).

The host bacterium *M. phosphovorus* and its lytic bacteriophages detected here were both indigenous in the activated sludge, as the sludge had been acclimatized for about six months prior to the experimental period with sterilized synthetic wastewater. As per the result in Fig. 1, during the first 12 days, the population of the phage-host system was steady against each other. The populations were approximately 8.5%–10% (FISH/DAPI) for *M. phosphovorus* and approximately 30–200 PFU/ml for the lytic bacteriophages. Afterward, by day 18, *M. phosphovorus* rapidly decreased to less than 1% while its lytic bacteriophages increased to more than 1,400 PFU/ml in a reverse tendency. After day 18, *M. phosphovorus* recovered in its population to approximately 5%–7.5%, while the lytic bacteriophages decreased to the level of approximately 10–100 PFU/ml. The FISH-positive cells are shown in time-sequence in Fig. 2. The increase and the decrease of the phage-host system's populations were apparently contrasted during the monitoring. Considering the phage-host relationship, the increase-decrease event of the phage-host system even in the activated sludge is no surprise. However, the present study is the first case study to monitor the population fluctuation of a bacterial host and its lytic bacteriophages, both indigenous in activated sludge.

In order to more precisely analyze the observed behavior of the phage-host system, the quantitative relationship

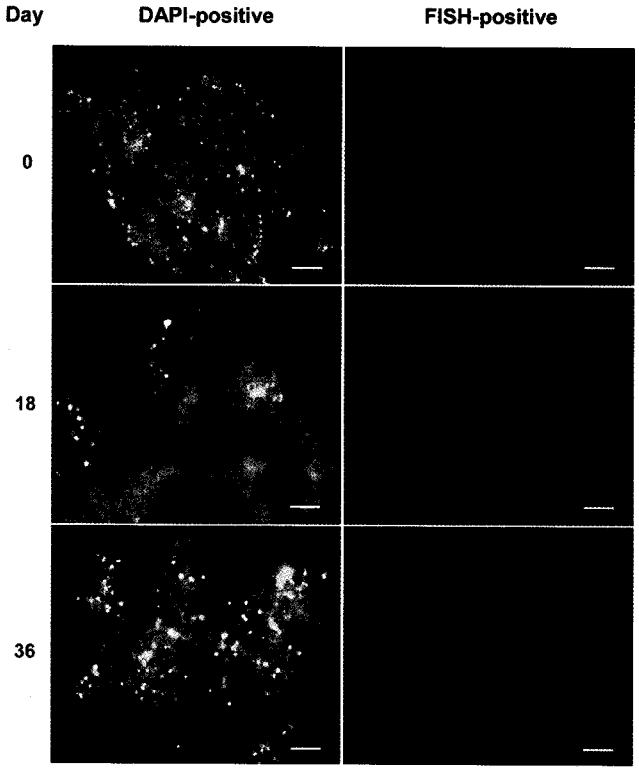

Fig. 2. FISH-positive *M. phosphovorus* cells (red) among DAPI-positive cells (blue) during the process operation.
FISH by *M. phosphovorus*-specific oligonucleotide probe MP2 (Cy3 labeled). Pictures were taken at ×1,000 final magnification (1/300–1/100 s exposures). Scale bar=20 μm.

between the bacterial host and its lytic bacteriophages is of interest. Suppose that the whole bacterial population in the activated sludge mixed liquor is in the order of 10^9 (cells/ml). During the period from day 12 to day 18 (Fig. 1), *M. phosphovorus* reduced from 10% of the DAPI area to less than 1%. Thus, the reduction of *M. phosphovorus* in the reactor during the six days is estimated to be around 2.5×10^{11} cells as the reactor volume is 2,500 ml. If there is no growth of *M. phosphovorus* during the six days of operation with the SRT of nine days, its population will decrease by around half, as $(8/9)^5=0.55$. Thus, the approximately half of the reduction of 2.5×10^{11} cells could be attributed to the excess sludge removal, and the rest could be attributed to the lysis by the bacteriophages. During the same period, the discharged amount of the bacteriophages from the process is estimated to be approximately 5×10^7 PFU [1,250 (ml)×6 (cycles)×6 (days)×10^3 (PFU/ml)], if the discharged amount of the bacteriophages was considered to be the same in every cycle as 10^3 PFU. So, during the period, the total number of phage particles released into the bulk of the activated sludge mixed liquor is estimated to be in the order of 10^8 (PFU). If the burst size of the bacteriophages is in the order of 10^2 (phage particles per host cell), then the number of lysed bacterial host cells is calculated to be approximately 10^6 cells during these six days. Thus, there is a big gap of 10^5 orders between the estimated reduction of the host population (10^{11} cells) and the estimated number of the bacterial host cells lysed (10^6 cells). The host reduction was far more than the number of estimated cells lysed. In addition, during the period from day 18 to day 36 (Fig. 1), theoretically the populations of *M. phosphovorus* and its lytic bacteriophage should remain at least at the same levels of those on day 18, respectively. However, their population dynamics were conflicting with the theoretical expectation. It is a very interesting observation, but there is no reasonable explanation for that at present. Nevertheless, the discrepancy in the quantitative comparison could be explained if we suppose that a large part of the phage particles released was physically trapped in the matrix of activated sludge flocs [12]. On the other hand, in the present study, the possibility that factors other than bacteriophages, such as predation by protozoa, caused reduction of host bacteria cannot be excluded [12], even though the predation by protozoa only specific to *M. phosphovorus* does not seem to be probable yet.

As an important role of bacteriophages in aquatic environments, viral-lysis-related bacterial mortality has been considered. For example, approximately between 10% and 20% of heterotrophic bacterial cells are destroyed by viral lysis each day in marine environments [18]. The control of bacterial community composition by the presumed species specificity and density dependence of viral infection is also considered to be one of their roles. It is apparently possible that the impact of viral infection on the mortality of host populations is incidental to the important role of phages in aquatic microbial communities for maintenance of the bacterial community structure [4]. For an activated sludge environment, an aquatic environment, Ogata *et al.* [15] and Lee *et al.* [12] reported the possibility that bacteriophages could influence the bacterial flora in activated sludge; however, no study on the relationship between the population dynamics of the bacterial host and its lytic bacteriophages, both indigenous in activated sludge, has been reported. The present study reported evidence for the involvement of *M. phosphovorus*-lytic bacteriophages in the decrease of the *M. phosphovorus* population, both indigenous, in the laboratory-scale activated sludge process (over 90% in maximum decrease from day 15 to day 21) (Fig. 1). This observation, in terms of phage-host ecology in activated sludge, suggests that lytic bacteriophages should be considered as one of the biological factors that can affect the bacterial community and structure in an activated sludge process. However, for a complete understanding of the correlation between the population dynamics of host bacteria and their lytic bacteriophages, replications of the host-decrease-and-its-phage-increase event in full-scale activated sludge processes, which are retaining a typically higher diversity of bacteria and bacteriophages, are required.

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