J. Microbiol. Biotechnol. (2006), **16**(10), 1518–1522

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Dynamics Behavior of Phage-Host System Related to *Microlunatus phosphovorus* in Activated Sludge with Host Inoculation

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Received: March 30, 2006
Accepted: May 14, 2006

Abstract In the present study, it was observed how the phage-host system that is naturally reproduced in activated sludge is affected by the host inoculation. The system of *Microlunatus phosphovorus* and its phages was selected as the phage-host system native to an activated sludge system operated for 19 days under sequencing anaerobic-aerobic conditions with glutamate as the main carbon source. The phage-host system related to *M. phosphovorus* was monitored by plaque assay for the phages and by fluorescent *in situ* hybridization (FISH) for the bacterial host. In addition, the whole phage structure was also monitored by pulsed-field gel electrophoresis (PFGE). During the first 9 days, the phage-host system was more or less steady at approx. 9% (FISH/DAPI) for *M. phosphovorus* and approx. 10,000 PFU/ml for its lytic phages. *Microlunatus phosphovorus* JCM9379 was inoculated into the activated sludge on day 10. Right after the inoculation, *M. phosphovorus* was approx. 24% (FISH/DAPI) whereas its lytic phages dropped down to approx. 500 PFU/ml. After the host inoculation (within 9 days), however, the phage-host system eventually reverted to its original level in each population. On the other hand, the whole phage structure was not significantly changed by *M. phosphovorus* inoculation but stable throughout the process operation. Only the minor change that four phage groups gradually became abundant after the host inoculation was observed.

Key words: Phage-host system, activated sludge, phage structure, *Microlunatus phosphovorus*

Bacteriophages, or phages in short, are viruses that are parasitic to bacteria as the host and lyse them. In some cases of marine and freshwater bacterial communities, the emergence of phages has been shown to cause the apparent lysis of almost entire bacterial populations [3, 4]. Suttle and Chan [19], and Cottrell and Suttle [5] suggested that the abundances of host bacteria and phages were positively correlated. In the cases of activated sludge bacterial communities, Ewert and Paynter [6], and Khan *et al.* [10] reported phages infectious to activated sludge bacteria. The ecology of the activated sludge phages has been also reported by Ogata *et al.* [16] and Hantula *et al.* [7]. From the reports, it is clear that there are phages in activated sludge. Recently, Khan *et al.* [11] and Lee *et al.* [13] reported on phages lytic to the bacteria that are naturally reproduced in activated sludge.

Lee *et al.* [14] have found two lytic phages specific to *Microlunatus phosphovorus*, which were isolated from activated sludge in where both the phage and the host were thought to have been naturally reproduced [15]. Here, the presumption that lytic phages may affect the bacterial community in activated sludge [13] deserves to be studied. In the present study, in order to understand the ecological role of phages to the host bacterial groups in activated sludge, an investigation on the population dynamics of the phage-host system related to *M. phosphovorus* according to the artificial boost of the bacterial host was conducted. A laboratory activated sludge process was operated, and then, *M. phosphovorus* was inoculated into the middle of the process operation. During the process operation, the population dynamics of the phage-host system and the whole phage structure dynamics were simultaneously monitored.

MATERIALS AND METHODS

Laboratory-Scale Activated Sludge Process

A laboratory-scale enhanced biological phosphorus removal (EBPR) activated sludge process was operated under the

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sequencing anaerobic and aerobic conditions for 19 days. One cycle of the sequence was comprised of a 30-min feeding phase, 90-min anaerobic phase, 150-min aerobic phase, 60-min settling phase, and 30-min 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 sodium glutamate·H_2O 314 mg, yeast extract 28 mg, $CaCl_2 \cdot 2H_2O$ 12.3 mg, $MgCl_2 \cdot 6H_2O$ 127 mg, KCl 58.8 mg, NH_4Cl 24.6 mg, $(NH_4)_2SO_4$ 30.2 mg, K_2HPO_4 18.2 mg, KH_2PO_4 19.6 mg, and allylthiourea (ATU) 5.6 mg, 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 sludge retention time (SRT) was maintained at 9 days. The seeding sludge was from a similarly operated SBR, which had been operated also with glutamate as the main carbon source under the sequencing anaerobic-aerobic condition for 6 months. The very original seed sludge for this SBR was from an urban wastewater treatment plant in Tokyo, Japan.

Bacterial Culture

M. phosphovorus JCM9379 obtained from Japan Collection of Microorganisms (JCM) was incubated at 25°C in the medium equivalent to 36-times-concentrated synthetic wastewater. On day 10 of the process operation, 100-ml culture of exponentially growing *M. phosphovorus* JCM9379 was inoculated (approx. 10% to sludge in weight). This bacterial strain and medium were also used for quantification of *M. phosphovorus*-lytic phages.

Chemical Analysis

Dissolved organic carbon (DOC) was analyzed by a TOC analyzer (TOC-V, Shimadzu, Japan). Mixed liquor suspended solids (MLSS) was analyzed according to *Testing Methods of Sewage* [8]. Phosphate was analyzed by an ion chromatograph (IC7000, Yokogawa Analytical Systems, Japan).

Quantification of *M. phosphovorus*-Lytic Phages

M. phosphovorus-lytic phages were counted by using the plaque assay method of Adams [1]. The 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, U.S.A.). 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* (OD around 0.6) 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*. After incubation, plaques were counted and then the concentration of *M. phosphovorus*-lytic phages was calculated as plaque forming unit (PFU). This quantification was carried out at every three days with duplicate testing.

Quantification of *M. phosphovorus*

Fluorescent *in situ* hybridization (FISH). The 1-ml activated sludge mixed liquor sampled from the end of the aerobic phase was centrifuged. 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.* [9] with minor modification. After thawing the sludge pellet in 1-ml 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 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'-GAGCAAGCTCTTCT-GAACCG-3') [9] labeled with Cy3 was obtained from Proligo (Proligo, Japan) 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].

Microscopy. Microscopy was performed with a fluorescent microscope Olympus BX51 (Olympus Optical, Japan) equipped with a CCD camera (DP-50, Olympus Optical, Japan). 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 Leica Qwin software version 2.3a (Leica Microsystems Imaging Solutions, U.K.).

Phage Structure Dynamics Analysis by Pulsed-Field Gel Electrophoresis (PFGE)

Phage DNA preparation. Every 3 days, 50 ml of the mixed liquor sample was collected at the end of the aerobic phase in a plastic conical tube during the process operation. To elute the phage particles from the activated sludge, the protocol by Kim *et al.* [12], which was originally developed for the elution of Poliovirus from activated sludge, was employed. Sodium nitrate ($NaNO_3$) was added to the sample at a final concentration of 1 M (pH 7.0) and then mixed for 10 min to elute the phage particles from the sludge sample. The mixture was centrifuged (1,000 ×*g*, 10 min) and then the supernatant was carefully transferred to a new sterilized tube. Bacteria and larger plankton in the supernatant were removed by filtration using a 0.2-μm pore-size filter. The filtrate was incubated in the mixture of DNase I and RNase A at 37°C for 30 min to degrade any

naked nucleic acid. After the nuclease treatment, 25 ml of the treated sample was transferred to a polycarbonate ultracentrifuge tube and centrifuged at 30,000 ×*g* (20°C, 80 min) in a Type 70 Ti rotor with Optima-L ultracentrifuge (Beckman, U.S.A.). The phage pellet was resuspended in 100 µl of 100× TE buffer and then purified using a Microcon-100 ultrafiltration unit (Millipore, U.S.A.) and stored at 4°C until use [17, 18].

The DNA of the purified phage particles was released by warming at 60°C for 20 min followed by cooling on ice. The DNA sample was handled carefully and pipetted as infrequently and slowly as possible to avoid shearing the high molecular weight nucleic acids liberated from the phage particles.

PFGE. The PFGE was performed as follows [17]. The phage DNA extracted was mixed with 1/10 volume of 10× loading buffer (TOYOBO, Japan) and applied to PFGE. PFGE was performed with the contour-clamped homogeneous electric field DR-III Cell (Bio-Rad) under the following conditions: 1% SeaKem GTG agarose (FMC, U.S.A.); 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. The molecular weight marker used was the lambda/HindIII restriction digest (Sigma, U.S.A.) or MidRange II PFG marker (New England Biolabs, U.K.). After electrophoresis, the gels were stained for 30 min in Vistra Green (Amersham-Pharmacia Biotech, U.S.A.) according to the manufacturer's instructions and digitally scanned for fluorescence by using a fluorescent image scanner, Fluor Imager (Molecular Dynamics, U.S.A.).

RESULTS AND DISCUSSION

Monitoring of the Phage-Host System

The results of the monitoring of *M. phosphovorus* and its lytic phages are plotted in Fig. 1. During the first 9 days, the population of phage-host system was more or less steady. The populations were approx. 8–9% (FISH/DAPI) for *M. phosphovorus* and approx. 5,000–10,000 PFU/ml for its lytic phages. In the present study, synthetic wastewater prepared from the sterilized medium diluted with tap water was used. *M. phosphovorus* was detected even before day 10 when it was inoculated into the activated sludge. Considering the concentration of biomass in the influent, *M. phosphovorus* was reproducing in the activated sludge feeding on the organic matter in the influent. The phages lytic to *M. phosphovorus* detected by the plaque assay method were also thought to be multiplying in the activated sludge, most probably being hosted by the natively growing *M. phosphovorus*. The phage-host relationship observed before day 10 implied that they had a rather stable coexistence.

On day 10, *M. phosphovorus* was inoculated with an expectation that dynamic behavior of the phage-host

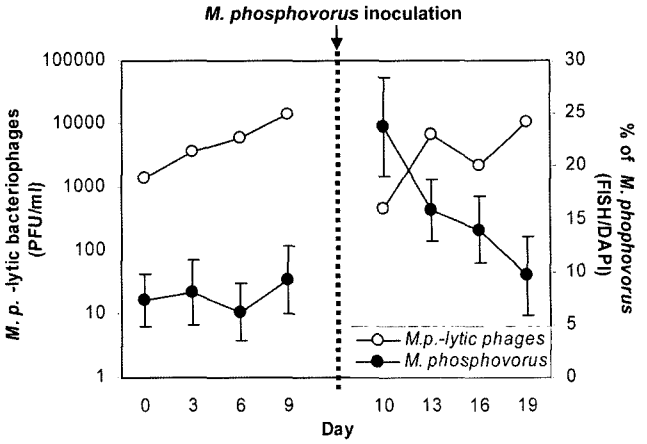

Fig. 1. Population dynamics of the phage-host system related to *M. phosphovorus* during the process operation.

system would be observed. Initially, the amount of *M. phosphovorus* was around 9% over the total DAPI-stained bacterial count. By its inoculation on day 10, it increased to 24% (FISH/DAPI). Then, within 9 days, it reverted to the original level, 9% (FISH/DAPI). On the other hand, the phage count, which was in the range of 1,000 to 10,000 PFU/ml before day 10, decreased to around 500 PFU/ml right after inoculation of the host, and then, it also reverted to its original level after day 13. Here, the point of interest was whether the reduction of *M. phosphovorus* after day 10 was mainly caused by lysis by its phages. Unfortunately, it is rather difficult to tell whether this was indeed the case. If the phages had mainly caused the reduction of *M. phosphovorus*, then surplus formation of phage particles should have been observed after day 10. However, the phage count went back only to its original level, and the evidence that phages mainly reduced *M. phosphovorus* was not observed. Nevertheless, it should be noted here that the sample applied for the plaque assay was only the supernatant of the activated sludge mixed liquor.

The reduction of *M. phosphovorus* during day 10 to day 19 can be partly attributed to excess sludge removal. As the SRT was maintained at 9 days, around 65% of the total *M. phosphovorus* can be eliminated if their growth is negligible. Indeed, the reduction of the phage population from 25% to 9% (FISH/DAPI) was just around 60%. However, as they were growing in activated sludge before day 9, their growth cannot be neglected. That is, the excess sludge removal only cannot explain the reduction of *M. phosphovorus*. It could be possible that they might have been grazed by protozoa or metazoa. In addition, RNA phages or single-stranded DNA phages that mostly resided in the floc part, not supernatant part, could have caused the reduction of the host, as these types of phages cannot be observed by the PFGE method in this study.

The decrease of the *M. phosphovorus*-lytic phages into the half level after the host inoculation might be mainly caused by their phage-host adsorption-infection, but the re-increase into their original level rather than to a level exceeding the original level could not be clearly explained from the results above. However, it could be anticipated that the phages were somehow involved in the host reduction, whereas sudden excess loading of bacterial host itself could not consequently affect its phage population in a short time. It could mean that the phage-host system in activated sludge is complementary and has a long-term relationship, if the system was not affected by other environmental factors. For a more complete understanding on the ecological rules of phage-host systems in activated sludge, this kind of studies including phage-host system for a long-term relationship under various conditions are deserve to be further investigated.

Monitoring of the Whole Phage Structure Dynamics

The whole double-stranded DNA phages was monitored during the process operation by PFGE, and no significant dynamic behavior by the host inoculation was observed (Fig. 2). The total number of the phage DNA bands in the gel was not significantly changed along with the operating time. However, four phage groups (circle-end arrows; 24 kb <DNA size <73 kb) gradually became abundant after day 10 of the host inoculation. On the other hand, most of the other phage groups were stable, and especially the particular phage group (dotted arrow; 24 kb< DNA size <48.5 kb) was stable and abundant during the entire process operation with no effect by *M. phosphovorus* inoculation (Fig. 2).

The PFGE result gives supplementary insight into the behavior of total double-stranded DNA phages in activated sludge flocs, as the phages assayed here were eluted from activated sludge flocs and then applied to PFGE. However, here again, no bands to be intensified were found after day 10. Lee *et al.* [14] reported two double-stranded DNA phages, ϕMP1 and ϕMP2: both are lytic to *M. phosphovorus*. The sizes of these phages are estimated to be 42 to 48 kb for ϕMP1 and 21 to 28 kb for ϕMP2, and their latent periods were 18 h and 36 h, respectively. Because these phages are double-stranded DNA phages, their sizes within the

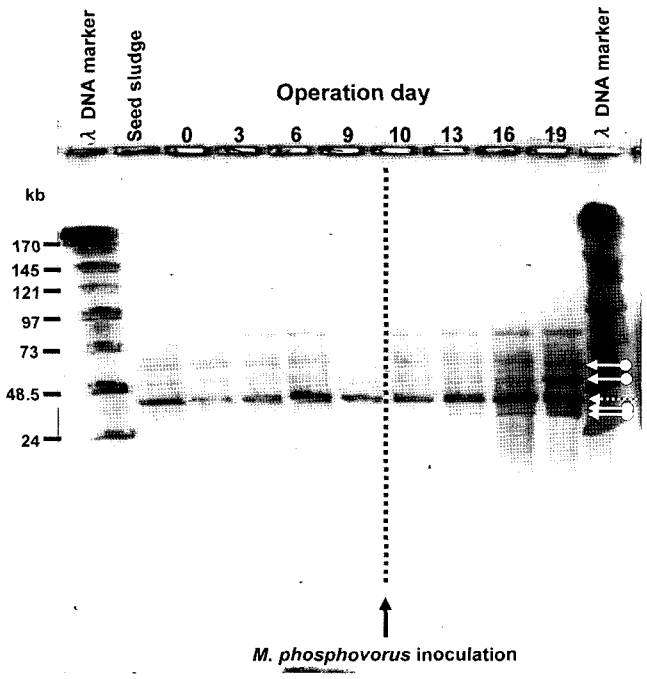

Fig. 2. PFGE analysis of the whole phage structure dynamics during the process operation.

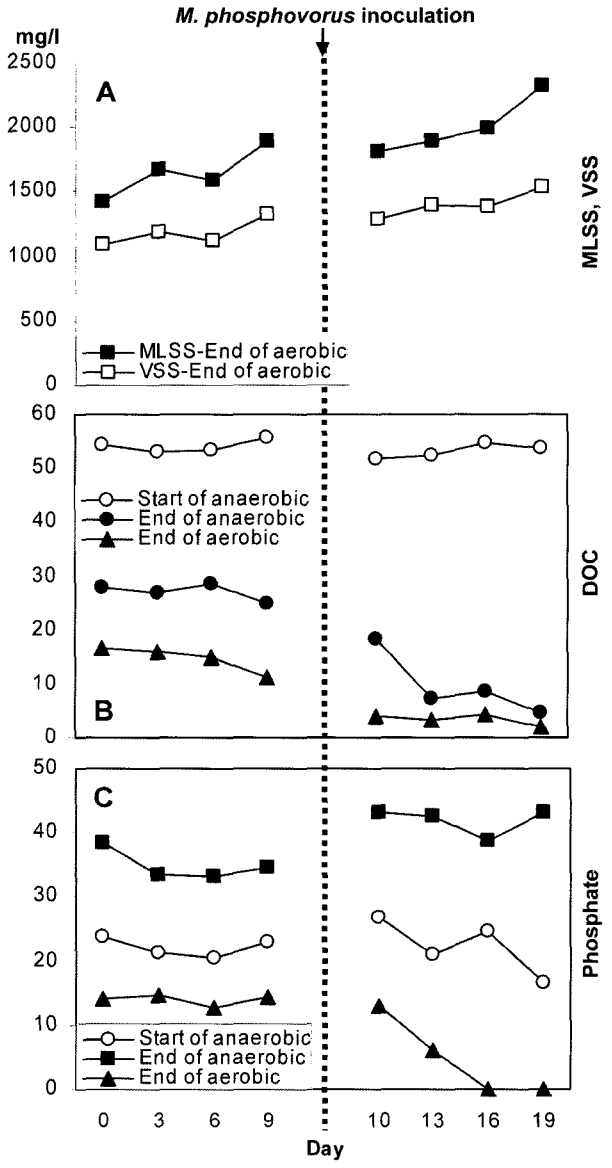

Fig. 3. The treatment performances of the activated sludge process.

detectable range by the PFGE condition employed here, and their latent periods short enough for the sampling interval of 3 days, the bands corresponding to these phages should have been intensified if they lysed their host. However, no clear narrow bands were observed in these size ranges nor any bands to be intensified after the inoculation of *M. phosphovorus*. However, it might be expected that the five phage groups, indicated by arrows in Fig. 2, that became more abundant by the host inoculation, were *M. phosphovorus*-related phages, and if so, they possessed broad host range to infect *M. phosphovorus*.

Treatment Performances

The treatment performances of the activated sludge process are shown in Fig. 3. The removal of phosphate was improved after day 10 when *M. phosphovorus* was inoculated (Fig. 3C). From the results, no clear influence of phages on the performance of the wastewater treatment process was found. However, the authors would like to emphasize that there are phages that are related to bacteria in activated sludge, as were clearly observed in this study. Because phages are able to lyse their host, then theoretically, they can affect their host bacteria. Consequently, if the host bacteria were playing an important role in wastewater treatment, then the treatment performance could be affected by bacteriophages. More careful study would be needed to clarify the relationship between phages and their host bacteria in activated sludge.

Acknowledgment

S. H. Lee was supported by a JSPS (Japan Society for Promotion of Science) Research Fellowship (JSPS2003-11439) during the course of this work.

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