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Isolation, Physiological Characterization of Bacteriophages from Enhanced Biological Phosphorus Removal Activated Sludge and Their Putative Role

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Abstract This study aims at characterizing the bacteriophages isolated from activated sludge performing enhanced biological phosphorous removal (EBPR) to understand the interactions between the phage-host system and bacterial community. Sixteen bacterial isolates (E1-E16) were isolated as host bacterial strains from EBPR activated sludge for phage isolation. Forty bacteriophages based on their plaque sizes (2 plaques on E4, 4 on E8, 11 on E10, 5 on E14, 18 on E16) were obtained from filtered supernatant of the EBPR activated sludge. Each bacteriophage did not make any plaque on bacterial strains tested in this study except on its own host bacterial strain, respectively, indicating that the bacteriophages are with narrow host specificity. However, fourteen of the forty bacteriophages obtained in this study lost their virulent ability even on their own host bacteria. All of the lytic phages showed similar one-step growth patterns and had long latent period (about 9 hours) to reproduce their phage particles in their host bacterial cells. On the other hand, their probable burst sizes (6 to 48 per host cell) were large enough to actively lyse their host bacterial cells. Therefore, it could be implied that bacteriophages are also important members of the microbial community in EBPR activated sludge, and lytic phages directly decrease the population size of their host bacterial groups in EBPR activated sludge by lysis.

Key words: Activated sludge, microbial community, bacteriophage, EBPR, phage-host system

Activated sludge processes with the anaerobic/aerobic cycling of biomass have been employed widely for the removal of phosphorus (P) from wastewater [19, 26]. Many studies have been performed to clarify how the enhanced biological phosphorus removal (EBPR) is achieved including chemical transformation in the biomass of an EBPR activated sludge process [18, 19, 29]. EBPR is achieved by encouraging the accumulation of P in bacterial cells known as phosphate accumulating organism (PAO) in the form of polyphosphate (poly-P) granules, and the EBPR under particular plant operational conditions is reasonably well understood, even though how these conditions encourage the accumulation of poly-P in PAO is not clearly understood [3, 19, 26]. In order to understand the fundamental mechanisms of EBPR, many microbial ecologists and water environmental engineers have made efforts to analyze the bacterial communities in EBPR activated sludge by such methods as quinone analysis and 16S rRNA gene sequencing analysis. As a result, outstanding findings have been made within recent years, such as on *Proteobacteria* beta subclass, especially *Rhodocyclus*-related noble bacterial group as a PAO or the bacteria responsible for EBPR [5, 11, 13, 19, 24, 26].

On the other hand, according to the report by Jenkins and Hermanowicz [12], although P concentrations in the influent will vary with its source, most plant effluents should contain less than 1 mg/l P to protect the water environment from global problems such as eutrophication caused by high concentration of phosphorus and nitrogen. However, it is often not easy to maintain good P removal even at the EBPR activated sludge processes, and occasionally unexpectedly high P concentration (>1 mg/l P) is found in the treated water. Furthermore, the microbiological findings are rather on bacterial population and structure, and do not give direct input for instability of the bacterial community of EBPR activated sludge which might result in instability of the treated water quality especially related to P concentration.

In the present study, bacteriophages are of interest. Bacteriophages from the soil environment [2, 30, 31] and the marine environment [7, 10, 14] have been reported as an active member of microbial communities in the environments. However, even though bacteriophages from activated

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sludge process have been reported as a component of the microbial communities [6, 8, 9, 17, 23], they have not been critically considered as an important member of the communities even in recent review reports about the microbiology in EBPR activated sludge systems [19, 26]. On the other hand, recently, many microbial ecologists and water environmental engineers have presumed that lysis of certain bacterial groups including PAOs in EBPR activated sludge by bacteriophages might be one of the causes of inefficient P removal in the process. Khan *et al.* [15] newly reported the existence of both lytic and lysogenic phages in EBPR activated sludge. In spite of the report, there is still no clear understanding of the magnitade of bacteriophages' influences on bacterial communities in EBPR activated sludge because of the very limited studies in this respect and the seemingly complex nature of the bacteriophages.

Therefore, the present study paid attention to bacteriophages in the EBPR activated sludge process and reported new bacteriophages from EBPR activated sludge. Their physiological characteristics were also investigated for a more clear understanding of the role of bacteriophages on the bacterial community in EBPR activated sludge.

MATERIALS AND METHODS

EBPR Activated Sludge

A laboratory scale sequencing batch reactor (SBR) was operated, from which host bacteria and phage were isolated. The reactor was operated under the sequencing anaerobic and aerobic conditions to promote EBPR. One cycle of the SBR consisted of 30 min of feeding, 90 min of anaerobic phase, 150 min of aerobic phase, 60 min of settling, and 30 min of discharge. In each cycle, 0.1 l of feeding substrate with its composition shown in Table 1 and 5.9 l of tap water were added in the feeding phase, and 6 l of treated water was discharged in the discharge phage. The working volume of the SBR was 10 l, hydraulic retention time (HRT) was 10 h, and average solid retention time (SRT) was maintained for 15 days. In the feeding phase, dissolved oxygen was removed by purging with nitrogen gas. Automatically, pH was controlled between 7.0 and 7.2 with a pH controller. The original activated sludge taken from Shibaura wastewater treatment plant (WWTP) in Tokyo was employed as seeding sludge (average mixed liquor volatile suspended solid (MLVSS)=1,500 mg/l) for the SBR.

Table 1. Composition of feeding substrate.

Component	g/10 l	Component	g/10 l
$CH_3COONa(3H_2O)$	45.3	KCl	42.0
Yeast extract	92.3	$CaCl_2(2H_2O)$	8.8
Peptone	4.0	$MgCl_2(6H_2O)$	90.7
NH_4Cl	17.6	K_2HPO_4	18.0
$(NH_4)_2SO_4$	21.6	Allythiourea (ATU)	14.0

*Initial TOC=8,000 mg/l C; Initial P=640 mg/l P as final in the EBPR SBR. **20-fold dilution of this feeding substrate was used for both the broth and agar medium.

Bacterial Strain and Bacteriophage Isolation

For the isolation of bacterial strains, 10 ml of the EBPR activated sludge was precipitated by centrifugation at 8,000 rpm for 10 min, then the pellet was vigorously resuspended in 10 ml of saline solution (0.9% $NaCl_2$, pH 7.0) by use of a vortex mixer. This stage was repeated three times, then the last pellet resuspension was preincubated overnight in 20 ml broth medium (20-fold dilution of the feeding substrate). The preincubated resuspension was centrifuged at 1,500 rpm for 10 min and the supernatant was serially diluted in broth medium. These dilutions were spread on the agar medium (1.5% agarose in the broth medium) and incubated at 25°C for 3 days. Different colonies based on their sizes, colors, and shapes were reisolated three times to ensure the purity of the bacterial isolates. Each bacterial isolate was then employed as a host bacterial strain for phage isolation from the EBPR activated sludge. Bacterial isolates were stored in 30% glycerol stocks at - 80°C.

In order to isolate the bacteriophages that lyse each bacterial strain, 50 ml of the activated sludge was taken at the middle of the aerobic phase of EBPR SBR three times on different days. Each activated sludge sample was centrifuged at 8,000 rpm for 15 min and the supernatant was filtered with 0.45-μm-pore-size membrane filter. Each filtrate (1 ml) was directly mixed with 1 ml of each exponentially growing bacterial isolate in broth medium supplemented with 0.5% $CaCl_2$ ($CaCl_2 \cdot 2H_2O$, Waco Chem. Co., Japan) and incubated for 20 min at 25°C to allow phage absorption before mixing with 2 ml of soft agar (0.7% agarose in the broth medium containing 0.5% $CaCl_2$). The mixtures were poured over the agar medium supplemented with 0.5% $CaCl_2$ to form a thin layer. The plates were incubated upside down at 25°C for about 3 days to observe any kind of plaques on the lawn of each strain. After incubation, clear single plaques were picked from the plates based on their size, and each individual plaque was reisolated three times by serial plaque formation to ensure the purity of the phage isolate [1]. Each phage lysate was produced by liquid infection of individual bacteriophage from the third purified single plaque on its own host strain grown in broth medium with 0.5% $CaCl_2$ [20]. Bacteriophage samples were stored at 4°C after treatment with 0.5% chloroform and filtration through 0.2-μm-pore-size membrane filters.

Host Range Test

All of the isolated phages were subjected to host range test with the sixteen bacterial isolates obtained in this study, *Microlunatus phosphovorus* JCM9379, *Gordonia amarae*

JCM3171, and *Acinetobacter calcoaceticus* JCM6842. The bacterial isolates were obtained from the same microbial community as where the first bacteriophages were isolated. On the other hand, *M. phosphovorus*, *G. amarae*, and *A. calcoaceticus* were selected because they have been pointed out as candidates of polyphosphate accumulating organism (PAO) responsible for EBPR [19, 20, 25]. Each 10 µl of all titered phage resuspensions (10^8 to 10^{10} PFU/ml) was dropped on the preset lawn of each subjected bacterial strain in double-agar layer plates of 20-fold diluted feeding substrate containing 0.5% $CaCl_2$. All the plates were then incubated at 25°C for 3 days to check plaque formation [21].

Identification of host bacterial strains which supported plaque formation in this study was performed by sequencing the parts of their 16S rDNA using the MicroSeq 500™ 16S rDNA bacterial sequencing kit (Applied Biosystems, U.S.A.) and doing sequence similarity alignments of the sequences by BLAST search (www.ncbi.nih.gov/BLAST/).

One-Step Growth Experiment and Probable Burst Size

One-step growth of the phages was determined as described by Adams [1] with minor modifications. Briefly, 1 ml of overnight-cultured each host strain was transferred to 20 ml of broth medium and incubated with shaking for about 12 h, until A_{600} reached about 0.6 and viable cell counts were around 10^8 cells/ml. One-milliliter portion of each host culture was transferred to a fresh tube and mixed with each phage at a multiplicity of infection (MOI) of 1. Each mixture was incubated at 25°C for 20 min to allow phage absorption. After this absorption period, each mixture was diluted to 10^{-2} and 10^{-3} in 2 ml of broth medium. Each dilution was incubated at 25°C for 3 days under gentle mixing for phage amplification. Each incubated dilution was sampled of 100 µl at every 3-h-interval and then applied to plaque count test with its exponentially growing host bacterial strain on a double-agar plate. This experiment was performed three times and the average plaque count was plotted against incubation time to obtain the one-step growth curve of each phage. The average plaque number of each phage from 21st to 24th h was considered as its probable burst size.

Results and Discussion

Bacterial Isolates and Host Strains

Sixteen bacterial isolates (E1-E16) were obtained from the EBPR activated sludge for lytic phage isolation and they could grow well at the temperate range of 20°C to 30°C in both broth and agar medium of 20-fold diluted feeding substrate. Five bacterial isolates (E4, E8, E10, E14, E16) supported plaque formation with the EBPR activated sludge. The host bacterial strains that supported plaque formation were identified as shown in Table 2.

Of the sixteen bacterial isolates, the five identified host bacterial strains had similarity index higher than 90%. The affiliation of the five host bacterial strains are as follows, based on tentatively identified species: two of *Proteobacteria* alpha subclass, one *Proteobacteria* gamma subclass, and two *Flavobacterium*. No bacterial isolate which supported plaque formation belonged to the *Proteobacteria* beta subclass in this study, while previous studies reported that significant part of PAOs might belong to this group [5, 11, 13, 16, 19, 24, 26].

Table 2. Bacterial isolates and host bacteria identification.

Strain	Closest group[a]	Phylum
E1	Not identified	-
E2	Not identified	-
E3	Not identified	-
E4	Uncultured *Caulobacter* sp. clone GCPF7 (96%; 418/435) [AY129783]	Alpha subclass *Proteobacteria*
E5	Not identified	-
E6	Not identified	-
E7	Not identified	-
E8	*Flavobacterium columnare* strainB2 (95%; 453/475) [AJ491824]	Cytophaga
E9	Not identified	-
E10	*Flavobacterium columnare* strain LP8 (93%; 453/483) [AB015480]	Cytophaga
E11	Not identified	-
E12	Not identified	-
E13	Not identified	-
E14	*Bosea* sp. 7F (99%; 449/450) [AJ250800]	Alpha subclass *Proteobacteria*
E15	Not identified	-
E16	*Pseudoxanthomonas broegberinensis* strain B1616/1 (97%; 473/486) [AJ012231]	Gamma subclass *Proteobacteria*

[a]The parts of 16S rDNA sequences of the host bacterial strains were subjected to sequence similarity alignments by BLAST search (www.ncbi.nih.gov/BLAST/). *The accession numbers for the partial 16S rDNA sequences (approx. 500 bp) of the bacterial strains such as E4, E8, E10, E14, and E16 are AY488505- AY488509, respectively.

Table 3. Host range of the lytic phages.

Phages[a]	Bacterial strains[b,c]								Phages	Bacterial strains							
	E4	E8	E10	E14	E16	M	G	A		E4	E8	E10	E14	E16	M	G	A
φE4a	DP	-	-	-	-	-	-	-	φE14d	-	-	-	DP	-	-	-	-
φE4b	DP	-	-	-	-	-	-	-	φE14e	-	-	-	DP	-	-	-	-
φE8a	-	DP	-	-	-	-	-	-	φE16a	-	-	-	-	DP	-	-	-
φE8b	-	+	-	-	-	-	-	-	φE16b	-	-	-	-	+	-	-	-
φE8c	-	+	-	-	-	-	-	-	φE16c	-	-	-	-	+	-	-	-
φE8d	-	+	-	-	-	-	-	-	φE16d	-	-	-	-	DP	-	-	-
φE10a	-	-	DP	-	-	-	-	-	φE16e	-	-	-	-	+	-	-	-
φE10b	-	-	+	-	-	-	-	-	φE16f	-	-	-	-	+	-	-	-
φE10c	-	-	+	-	-	-	-	-	φE16g	-	-	-	-	+	-	-	-
φE10d	-	-	+	-	-	-	-	-	φE16h	-	-	-	-	+	-	-	-
φE10e	-	-	+	-	-	-	-	-	φE16i	-	-	-	-	+	-	-	-
φE10f	-	-	+	-	-	-	-	-	φE16j	-	-	-	-	+	-	-	-
φE10g	-	-	+	-	-	-	-	-	φE16k	-	-	-	-	+	-	-	-
φE10h	-	-	+	-	-	-	-	-	φE16l	-	-	-	-	+	-	-	-
φE10i	-	-	+	-	-	-	-	-	φE16m	-	-	-	-	+	-	-	-
φE10j	-	-	DP	-	-	-	-	-	φE16n	-	-	-	-	+	-	-	-
φE10k	-	-	+	-	-	-	-	-	φE16o	-	-	-	-	+	-	-	-
φE14a	-	-	-	DP	-	-	-	-	φE16p	-	-	-	-	DP	-	-	-
φE14b	-	-	-	DP	-	-	-	-	φE16q	-	-	-	-	+	-	-	-
φE14c	-	-	-	DP	-	-	-	-	φE16r	-	-	-	-	DP	-	-	-

+, Lysed; -, resistant; DP, disappeared plaque formation.
[a]Each phage from third plaque formation was used for host range test.
[b]All of the bacteriophages could not lyse the bacterial strains such as E1, E2, E3, E5, E6, E7, E9, E11, E12, E13, and E15 at all.
[c]M, *Microlunatus phosphovorus* JCM9379; A, *Acinetobacter calcoaceticus* JCM6842; G, *Gordonia amarae* JCM3171.

Bacteriophages

The filtered (0.45-μm-pore-size) supernatant of the EBPR activated sludge directly supported plaque formation on the lawn of the five bacterial isolates (36 PFU/ml with E4, 48 PFU/ml with E8, 161 PFU/ml with E10, 69 PFU/ml with E14, 1,155 PFU/ml with E16, as average values from the activated sludge samples taken on three different days). The PFU values were high enough even without the phage enrichment. It seems that bacteriophages are active as a member of the microbial community in EBPR activated sludge.

Forty selected bacteriophages (2 plaques from E4, 4 from E8, 11 from E10, 5 from E14, 18 from E16) based on their sizes (diameters, 0.5 to 5 mm) were purified by serial plaque formation and collected (10^8 to 10^{10} PFU/ml) by centrifugal ultrafiltration of the amplified phage solutions with an ultrafilter (Centriprep YM50, Amicon, Millipore, U.S.A.) to use for physiological characterization and investigation of their role in EBPR activated sludge.

Host Range of the Bacteriophages

Each collected phage (10^8 to 10^{10} PFU/ml) from each third purified single plaque did not lyse the other bacterial isolates obtained in this study, *M. phosphovorus* JCM9379, *G. amarae* JCM3171, and *A. calcoaceticus* JCM6842, except its own host bacterial strain (Table 3). In other reports, phages from soil [30] and from sludge [22] also showed host specificity on their host bacteria. It seems that the presence of host-specific lytic phage is also likely in EBPR activated sludge.

On the other hand, broad-host range of phages from EBPR activated sludge has been reported by simultaneously isolating host bacteria and phages, and investigating the host range of isolated phages [15]. In other reports, phages from soil [2, 31] and sludge [8, 9, 17, 27] also showed a broad-host range. Khan *et al.* [15] implicated that the covalent host infection of phages from the EBPR activated sludge might contribute to gene transfer in the bacterial communities. The host range of the phages obtained in this study is so narrow that gene transfer mediated by phage may not be important. However, contradictory reports have been reviewed and reported by Khan *et al.* [15]. Gene transfer by phages is a very interesting and important phenomenon for wastewater treatment. Thus, further and more careful studies are necessary to make any conclusion on host specificity of the phages.

Each 10 μl of titered (10^8 to 10^{10} PFU/ml) phage resuspensions from third purified single plaque was subjected to making plaque on the bacterial strains tested in this study. Unfortunately, all bacteriophages from E4 and E14, one of four bacteriophages from E8, two of eleven bacteriophages from E10, and four of eighteen

bacteriophages from E16 lost their virulent activity, even on their own host bacterial strains (Table 3). One of the possible reasons is the presence of a lysogenic relationship between host bacteria and bacteriophages [14]. Khan *et al.* [15] also reported the existence of lysogenic phages in EBPR activated sludge by showing the lysis failure by the bacteriophages on their original host bacteria from which they were first isolated.

It means that both lytic and lysogenic phages are also important members of bacterial groups composing the microbial community in EBPR activated sludge.

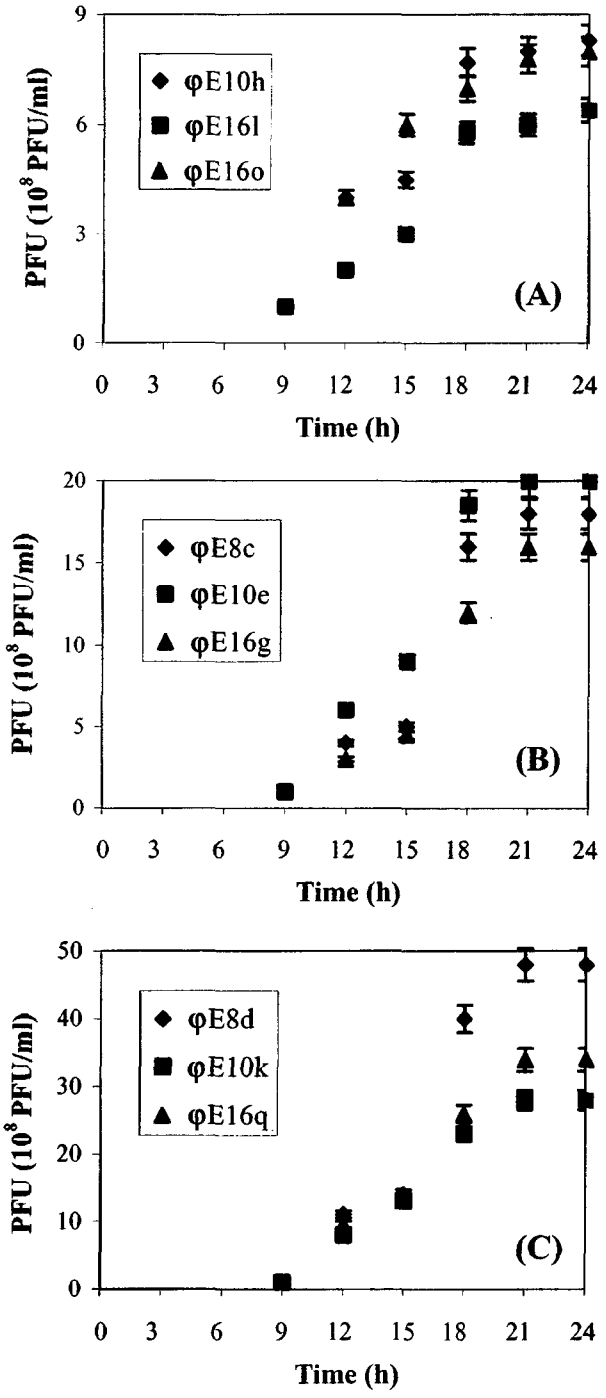

Fig. 1. One-step growth curves for the lytic phages from EBPR activated sludge.

One-Step Growth Curve of the Lytic Phages

The one-step growth curves of the lytic phages obtained in this study are shown in Fig. 1: those for φE10h, φE16b, φE16e-φE16f, φE16h, and φE16k-φE16o are represented on Fig. 1(A), those for φE8b-φE8c, φE10c-φE10d, φE10f, φE16c, φE16g, and φE16i-φE16j on Fig. 1(B), and those for φE8d, φE10b, φE10e, φE10g, φE10i, φE10k, and φE16q on Fig. 1(C).

All of the lytic phages showed typical and similar one-step growth patterns. The plaque count at the initial several hours would have been in the order of 10^8 PFU/ml, which is equivalent to the number of host cells infected by the initially inoculated phage. It was only after the 9th hour that quantitative monitoring of plaque formation was performed. After the 9th hour, the increase of plaque count was apparently observed, indicating the lysis of host cells infected by the lytic phage at the very beginning of the experiment and the release of phage particles into the media. The plaque count increased in the following couple of hours, indicating the continuation of the lysis followed by a plateau because most part of bacterial cells infected at the very beginning was lysed. Based on the observation, it can be concluded that the latent period was about 9 h for all bacteriophages tested.

The latent period observed here was considerably longer when compared with that of the phages from marine environment (1.5 to 3 h) [14] or soil environment (2 h) [28]. However, the latent period of 9 h is short enough to imply that the lysis of the lytic phages affects the abundance of the host bacteria when it is compared with the doubling time of their host bacterial strains. The doubling time of the host bacteria in the reactor is estimated to be in the order of days or longer when considering the SRT being 15 days.

Probable Burst Size of the Lytic Phages

The average plaque number of the triple one-step growth experiments was not actual burst size of each lytic phage in general, but it should be nearly equal to its burst size because the mixed ratio of each lytic phage over its host bacterial strain (multiplicity of infection; MOI) was 1. For the burst size of each lytic phage, the average plaque number of each lytic phage from 21st to 24th h in the triple one-step growth experiments was considered as its probable burst size, because the plaque number reached a maximum without a significant change during that period.

The probable burst size of each lytic phage is shown in Table 4. The lytic phages, φE8b, φE8c, φE10c, φE10d, φE10f, φE16c, φE16g, φE16i, and φE16j, had probable

Table 4. Probable burst size of the lytic phages.

Phages for E8	PB[a]	Phages for E10	PB	Phages for E10	PB	Phages for E16	PB	Phages for E16	PB	Phages for E16	PB
φE8b	12	φE10b	22	φE10h	8	φE16b	6	φE16i	18	φE16o	8
φE8c	18	φE10c	10	φE10i	24	φE16c	11	φE16j	14	φE16q	34
φE8d	48	φE10d	16	φE10k	28	φE16e	6	φE16k	12		
		φE10e	22			φE16f	6	φE16l	6		
		φE10f	18			φE16g	16	φE16m	8		
		φE10g	28			φE16h	8	φE16n	8		

[a]PB, probable burst size of the lytic phages as average value at from 21st to 24th h in triple one-step growth experiments.
*φE4a, φE4b, φE8a, φE10a, φE10j, φE14a -φE14e, φE16a, φE16d, and φE16p lost their virulent activity.

burst size of about 10 to 20 (Table 4). On the other hand, φE8d, φE10b, φE10e, φE10g, φE10i, φE10k, and φE16q had over 20 of probable burst size, while φE10h, φE16b, φE16e, φE16f, φE16h, φE16k, φE16l, φE16m, φE16n, and φE16o had below 10 (Table 4).

The value of probable burst size was broad even for lytic phages from the same host strain. The probable burst size (6 to 48) of the lytic phages obtained in this study was smaller than the burst size (7.8 to 240) reported for phages from marine environment [14], but larger than the burst size (13 to 17) reported for phage from soil environment [28]. However, the probable burst size of marine bacteriophages fell within the range of 5 to 610; according to Børsheim [4], the average burst size of marine bacteriophage is 185. Therefore, the probable burst sizes were also large enough to imply that the lytic phages could be an important member of the microbial community in EBPR activated sludge, which could significantly affect the abundance of their host bacterial groups by lysis. It means that the lysis of the host bacterial groups by lytic phages could negatively influence on the population size of the host bacterial groups in EBPR activated sludge. In other words, lytic phages could immediately decrease the portion of their host bacterial groups.

Based on the viewpoints of host range and probable burst size in the present study, we suggest that lytic phages, which are specific to their host bacterial groups, probably play a key role to control their host bacterial populations or to select bacterial groups composing bacterial community of EBPR activated sludge by actively lysing their host bacterial groups. However, for a complete understanding of the role of bacteriophages in EBPR activated sludge, more definitive identification of the bacteriophages remains to be carried out as well as physiological characteristics of the bacteriophages related to the bacteria that are responsible for EBPR.

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