Original article

Isolation and characterization of a virulent bacteriophage infecting *Acinetobacter johnsonii* from activated sludge

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Abstract

A double-stranded DNA phage named AJO1, infecting *Acinetobacter johnsonii*, which plays an important role in wastewater treatment, was isolated from activated sludge in a full-scale municipal wastewater treatment plant. Based on morphological taxonomy, AJO1, with an icosahedral head 55 ± 2 nm in diameter and a non-contractile tail 8 ± 2 nm in length, was classified as a member of the *Podoviridae* family. Bacterial infection characteristics were as follows: no polyvalent infectivity, optimal multiplicity of infection of 10^{-2} ; eclipse and burst size of 30 min and 51.2 PFU-infected cells $^{-1}$, respectively. It showed considerable infectivity under a neutral pH condition (pH 6.0–9.0) and relatively high temperature (55 °C). Whole-genome sequencing of AJO1 revealed a linearly permuted DNA (41 437 bp) carrying 54 putative open reading frames and 4 repeats. This is the first report of isolation of an *A. johnsonii* phage, whose bacteriophage distribution and population dynamics are not well known. The results of this study could contribute to subsequent research on the interaction between bacteriophages and their hosts during wastewater treatment. In addition, AJO1 may become a candidate for potential therapy against *A. johnsonii* infection in clinical applications, since this species is an opportunistic pathogen.

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Keywords: *Acinetobacter johnsonii*; Bacteriophage; Isolation; Bacterial infection; Activated sludge; Wastewater

1. Introduction

Activated sludge, which contains a wide spectrum of bacterial species, plays a key role in the transformation and removal of pollutants in wastewater [1,2]. Different bacterial species have different biological functions and wastewater treatment requires cooperation among them. For example, the cooperation of some heterotrophic bacteria is required for mineralization of organic pollutants [3] and cooperation of nitrifiers (such as *Nitrosomonas* and *Nitrobacter*) and denitrifiers is required for completion of nitrogen removal [4,5]. At the same time, competition between floc-forming bacteria and filamentous bacteria like *Microthrix parvicella*, *Nocardia nova* and *Gordonia terrae* is important for the stability of

activated sludge floc structures [6–8]. Thus, balanced bacterial composition of activated sludge is vital for maintaining the function of wastewater treatment plants (WWTPs). For example, phosphorus removal will deteriorate when glycogen-accumulating organisms (GAOs) outcompete polyphosphate-accumulating organisms (PAOs) [9], and the water-sludge separation performance will worsen when filamentous bacteria like *M. parvicella* become the dominant species [10]. Changes in wastewater composition, operational and environmental conditions, etc., have been considered the main reasons triggering disruption of bacterial composition balance in activated sludge [11–13].

On the other hand, an outbreak of bacteriophages could also be one of the possible reasons for disruption of community balance in activated sludge [14,15]. Thus, tracing dynamic changes in bacteriophages targeting important functional bacterial groups is helpful in elucidating root causes of malfunctioning of WWTPs. Recent studies revealed that

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phages and their associated ecological functions might drive and control the dynamics of several important bacterial groups, including PAOs and filamentous bacteria [16,17]. However, interactions between bacteriophages and their hosts are still not well understood, since over 90% of bacteriophage sequences detected from activated sludge are unknown [18]. It is thus urgent to acquire more bacteriophage isolates for better understanding interactions between important bacterial groups and their phages in WWTPs.

In this study, bacterial strains were isolated from activated sludge suffering sludge bulking in a WWTP in Beijing, China. *Acinetobacter johnsonii* has attracted much public attention due to its validated capacity for poly- β -hydroxybutyric acid synthesis, carbapenem resistance and co-aggregation in wastewater treatment [19–23]. At the same time, *Acinetobacter* spp., opportunistic pathogens with microbiological, clinical and epidemiological features, are present ubiquitously in nature and have been isolated from diverse habitats [24–27]. Therefore, *A. johnsonii* was used as the primary host bacteria for isolation of bacteriophages from activated sludge by the dual-plate method. A phage showing high specificity to *A. johnsonii*, named AJO1, was acquired and characterized, including its morphology, host range and burst size. In addition, potential functions were hypothetically described based on genome sequencing. This study is part of an effort to improve our understanding of the interactions between phages and their hosts in activated sludge.

2. Materials and methods

2.1. Bacterial strains used in study

A. johnsonii strain JN-11 was used as the primary host for phage isolation, propagation and characterization. This bacterium *A. johnsonii* and other strains, including *Escherichia coli*, *Kocuria rosea*, *Modestobacter versicolor*, *Flavobacterium*, *Bacillus methylotrophicus* and *Delftia tsuruhatensis*, were isolated from a full-scale WWTP in Beijing in March when sludge bulking occurred (sludge volume index, 180 ml g⁻¹). *A. johnsonii* could form 1–2 mm in diameter, milk-white, round colonies on Luria Bertani (LB) agar after 20 h at 30 °C. According to microscopic examination, *A. johnsonii* was in the shape of a short rod without flagellum, Gram-negative, and generally appeared in pairs or groups. In addition, other six *Acinetobacter* strains including *Acinetobacter nosocomialis*, *Acinetobacter lwoffii*, *Acinetobacter junii*, *Acinetobacter haemolyticus*, *Acinetobacter calcoaceticus* and *Acinetobacter baumannii*, used for host range tests, were collected from the China General Microbiological Culture Collection Center (CGMCC). Bacterial cultivation was performed in LB broth at 30 °C [28], and all strains were frozen at –20 °C and –80 °C with glycerol addition for short-term and long-term storage, respectively. Bacterial growth was monitored turbidimetrically by measuring optical density at 600 nm (OD₆₀₀), where an OD unit of 1.0 was equivalent to about 3 × 10⁸ cells ml⁻¹. The 16S rRNA genes were amplified according to standard PCR protocol with universal primers,

27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') [29]. Purified PCR products were sequenced directly and compared to GenBank Database using Basic Local Alignment Search Tool (BLAST).

2.2. Isolation and characterization of phage

A. johnsonii was chosen as the host strain for bacteriophage isolation. A 20 l activated sludge sample was centrifuged at 8000 × g for 15 min before being filtered through cellulose acetate membrane filters (0.45- μ m and 0.20- μ m pore sizes) to remove bacterial debris. Tangential flow filtration was used to further enrich the acquired filtrate. *A. johnsonii* phage enrichment was performed by adding ~10⁶ cells of *A. johnsonii* to 50 ml of LB broth and incubating with 1 ml of filtered activated sludge supernatant. Flasks were left for 1 h at room temperature without shaking to encourage phage absorption before further incubation with shaking at 30 °C for 6 h. After enrichment, the bacterial cells were first centrifuged at 8000 × g for 10 min and the supernatant was filtered through 0.22- μ m cellulose acetate membrane filters. A plaque assay was carried out to screen for the presence of lytic phage activity. An equivalent volume mixture of the filtered sample and host culture was added to melted LB agar and poured onto autoclaved agar. After incubating at 30 °C overnight, zones of lysis or plaque formation could be observed [30]. Plaques were purified through eight rounds of single plaque isolation to ensure that each plaque resulted from a single virion. Then, a single plaque formed on the *A. johnsonii* lawn was picked up in SM buffer (10 mM Tris–HCl, pH 7.5, 10 mM MgSO₄·7H₂O and 100 mM NaCl).

The host range of the phage was determined by the spot test method [31]. After purification, a dilution series of the phage (~10¹⁰ phages ml⁻¹) was spotted onto swabbed lawn plates of each bacterial strain and inspected for the presence of plaques. A one-step growth curve was drawn as described previously by Petrovski et al. [32].

2.3. Determination of multiplicity of infection (MOI)

MOI was defined as the ratio of virus particles to potential host cells [33]. To determine the optimal MOI, the host strain in the early exponential phase was cultured in LB broth at 30 °C. Four different ratios of purified phage stock (10⁻¹, 10⁻², 10⁻³ and 10⁻⁴) and a control solution were prepared in advance. After incubation at 30 °C, the samples in triplicate were taken from each MOI set and assayed to determine the phage titer. Viable cell counts were determined by plating samples on LB agar and then incubating overnight at 30 °C. The optimal MOI was the recommended mixing ratio for large-scale proliferation and enrichment of the bacteriophage.

2.4. Electron microscopy

Transmission electron microscopy [34] was performed to investigate phage morphology. After NaCl/PEG precipitation [16], the phage particles were free from host proteins and

could be directly used for electron microscopy. Virus particles were allowed to adsorb to formvar-coated 200-mesh copper grids for 10 min, then washed twice for 1 min in double-distilled water (ddH₂O). Virus particles were negatively stained with 2% (w/v) uranyl acetate for 5 min and allowed to air dry. Grids were examined under an H-7500 transmission electron microscope (Hitachi, Japan) at an accelerating voltage of 100 kV.

2.5. Phage stability under different conditions

pH stability and thermal stability tests were performed as previously described [35,36]. Phage stock was incubated in LB broths with different pH values in the range of 2.0–12.0 (1.0 pH intervals) at 30 °C. Similarly, phage stocks were mixed with LB media and incubated at 55 °C, 60 °C and 70 °C, respectively. Samples were taken at predetermined intervals and the surviving bacteriophages were immediately measured by the double-layer method.

A collimated-beam ultraviolet (UV) apparatus emitting monochromatic UV radiation at 254 nm was used for UV inactivation experiments. The phage solution was treated with UV for 10, 30, 60, 120 and 180 s, respectively, to evaluate the UV effect on phage infectivity. In order to avoid photoreactivation, dual-layer plates were inoculated under dark conditions.

2.6. Phage DNA extraction and sequencing of bacteriophage nucleic acids

Virions were precipitated using NaCl/PEG 8000, and then phage DNA was isolated using SDS/proteinase K as described previously [32]. Phage genomic DNA was sequenced using the MiSeq Sequencing Platform in Novogene Company (Beijing) and the sequenced reads were assembled using the SOAPdenovo strategy with a minimum of 50 × physical clone coverage [37].

Endonucleases including DNase I (Sigma), RNase A (Sigma) and Mung Bean Nuclease (New England Biolabs) were chosen to determine the nucleic acid type of the isolated phage at 37 °C, followed by agarose gel electrophoresis [38]. The working concentration and condition of these endonucleases is 1 unit μl^{-1} .

2.7. Genome annotation

The genome of the phage was annotated using the GeneMarkS interface (<http://topaz.gatech.edu/>), followed by manual inspection of all gene predictions. Putative open reading frames (ORFs) longer than 100 nucleotides were predicted using GeneMarkS and MetaGeneAnnotator (<http://metagene.cb.k.u-tokyo.ac.jp/>).

Sequence similarity was analyzed using the BLAST X interface against a non-redundant database, including data sourced from the NCBI and Protein Data Bank (PDB) databases, using a significance cutoff E-value of 1e^{-3} . Putative functions were assigned to the corresponding gene products

based on the results of a BLAST protein search [39,40]. The conserved domain database (CDD) (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>) and Pfam database (<http://pfam.sanger.ac.uk>) were used to identify conserved motifs and make predicted protein family allocations [41]. The presence of putative tRNA and transfer mRNA (tmRNA) was screened using RNAmmer [42] and tRNAscan-SE [43,44] and sRNA genes were screened using Rfam [45]. Predicted promoter regions were identified using neural network promoter prediction [46]; and putative terminator structures were identified using the web tool FindTerm (<http://linux1.softberry.com/berry.phtml>). A phylogenetic tree was also constructed and displayed using MEGA7 [47] via the neighbor-joining method [48].

3. Results and discussion

3.1. Phage isolation and characterization

A lytic bacteriophage specific to *A. johnsonii* (named AJO1) was isolated from activated sludge in a municipal wastewater treatment plant. After serial infection of *A. johnsonii*, several distinct, clear and round-shaped plaques with a diameter of around 4 mm were observed on the agar plate, suggesting that AJO1 was a typical virulent phage. To date, more than 5500 bacteriophages have been examined and classified into 13 families, mainly according to their overall morphological characteristics (tailed, polyhedral, filamentous and pleomorphic) since the introduction of negative staining in 1959 [49–52]. Electron microscopy (Fig. 1) showed that the isolated *A. johnsonii* phage AJO1 had collar and tail structures, morphologically appearing to fall into the order Caudovirales [53]. The structural features of AJO1, with an icosahedral head 55 ± 2 nm in diameter and a short non-contractile tail 8 ± 2 nm in length, were consistent with morphotype C1 of the *Podoviridae* family [54], which accounts for only 13.9% of the tailed phages, including two other families, *Siphoviridae* (61.7%) and *Myoviridae* (24.5%) [55], according to the International Committee on Taxonomy of Viruses [56]. Phages in the *Podoviridae* family are typically virulent and have been well documented in various

Fig. 1. Transmission electron micrograph of phage AJO1.

environments, including food [57,58], marine sediment [59], hospital sewage [60] and activated sludge [56,61].

The infection cycle of AJO1 was characterized by the one-step growth curve test as shown in Fig. 2. The eclipse and rise periods of AJO1 were 30 min and 40 min, respectively and the burst size of phage AJO1 was 51.3 phages per infected cell⁻¹. In general, most *Podoviridae* phages have an eclipse period between 10 and 110 min [57,62,63], while the burst sizes vary between 22 and 801 phages per infected cell⁻¹ [62,64,65]. The relatively short eclipse period and small burst size of AJO1 indicate its ability to produce sufficient virions within a short time to infect the host bacteria [60,66].

The optimal MOI of AJO1 was investigated by incubating mixtures of bacteria and phages with different ratios, as shown in Supplementary Fig. 1. Phage infection occurred after 20 min, and the number of bacteria decreased dramatically under the ratio of phage to bacteria of 1:100. The optimal MOI of AJO1 was thus determined to be 10⁻², which was proposed as the optimized mixture ratio used in subsequent experiments.

The optimal MOI values of *Podoviridae* phages vary from 10⁻³ to 10 [57,60,67,68]. Different bacteriophages could have different optimal MOI values within the same family, even against the same host bacteria. For example, optimal MOI values of AB1 and AP22 infecting *A. baumannii* were 10⁻⁴ and 10⁻³, respectively [17,59]; those of PaP2 and PaP1 against *Pseudomonas aeruginosa* were 10 and 10⁻² [67,69]. It has been considered that the diverse forms and structures of phages are key factors affecting phage adsorption efficiency, resulting in the differences in optimal MOI values.

As shown in Supplementary Table 1, no strain among the 14 tested species, including *A. nosocomialis*, *A. lwoffii*, *A. junii*, *A. haemolyticus*, *A. calcoaceticus* and *A. baumannii* belonging to the *Acinetobacter* genus, was found susceptible to phage AJO1, except for *A. johnsonii*, showing its strict host specificity, though virulent phages have been reported to be able to infect more than one bacterial host across genera and family and even across a phylogenetic phylum [17,66,70,71]. *Podoviridae* phages have usually been found to exhibit a

narrow host range; for example, phage Φ A318 had a limited host range within the *harveyi* group [68], and phages Φ 22 and Acibel007 exhibited strict specificity to *Weissella cibaria* N22 [57] and *A. baumannii* [66], respectively. The recognition between phage structure proteins and host receptors probably influenced the phage host range [72]. The presence of specific antigens on the host cell surface has been speculated to be an important reason for the host specificity of most virulent phages [73].

3.2. Stability under different conditions

The effect of pH on phage growth was investigated, as shown in Supplementary Fig. 2. Optimal pH was 7.0. However, infective efficiency was over 70% over a pH range from 6.0 to 9.0. Different bacteriophages may have differing optimal pH conditions for infection, while observations have shown that the neutral pH condition is suitable for the physical stability of most phages [35,64,74]. Kerby et al. [75] found that the optimal pH for phage T7 belonging to the *Podoviridae* family was from 6.0 to 8.0 for long storage.

Preliminary testing showed that phage AJO1 stock solution retained almost 100% infective activity after incubation at 42 °C (data not shown). Higher temperatures of 55 °C, 60 °C and 70 °C were chosen to test the thermal stability of phage AJO1. As shown in Supplementary Fig. 3, the titers of phage AJO1 slightly dwindled to 5.31×10^7 phages ml⁻¹ after heating at 55 °C for 20 min, and more than 90% of phages lost their infective ability when the temperature rose to 60 °C. Thus, AJO1 could tolerate a relatively high temperature around 55 °C, which was similar to other reported *Podoviridae* phages [68].

The survival rate of AJO1 was tested under UV irradiation of 254 nm (see Supplementary Fig. 4). The phage almost died out under UV irradiation for 2 min, showing the high sensitivity of AJO1 to UV irradiation. It was reported that T7, a representative phage belonging to the *Podoviridae* family, was inactivated most rapidly in surface water at room temperature

Fig. 2. One-step growth curve of phage AJO1.

compared with other tested coliphages [76]. Even though there were slight differences among the inactivation kinetics of various phages by UV, the inactivation rates (k -values) of most pathogenic viruses were of the same order of magnitude [2]. Combined with previous investigations, it could be concluded that UV could be used as an efficient but non-specific approach to inhibiting phage activity.

3.3. Genome sequencing and annotation

According to gel electrophoresis results (see [Supplementary Fig. 5](#)), there were clear non-degraded bands in lane 1 (RNase A treatment) and lane MB (mung bean nuclease treatment), while in lane 2 treated with DNase I there was no band. Therefore, nucleic acid of AJO1 was degraded by DNase I instead of RNase A, indicating that its nucleic acid type was DNA. Mung bean nuclease, a single-strand-specific nuclease, did not degrade phage nucleic acid, showing that it was double-stranded. In conclusion, the nucleic acid of phage AJO1 was double-stranded DNA (dsDNA) [77].

The complete genome of *A. johnsonii* bacteriophage AJO1 was 41 437 bp, with a GC content of 41.15% ([Fig. 3](#)). A total of 54 ORFs were identified, but no potential tRNA coding genes were detected in the genome. A total of 22 ORFs (40%) were similar to other known sequences, whereas 32 ORFs produced no significant hits in database searches. Based on putative functions listed in [Table 1](#), the annotated ORFs of AJO1 could be categorized into five functional modules, including DNA replication, RNA metabolism, lysis, structure and additional functions. The phage genome encoded phage structure proteins, including a head-tail connecting protein, capsid protein and several tail proteins, including a tail tubular protein, tail fiber protein and tail collar domain protein. In addition, DNA replication proteins (adenylation DNA ligase

and primase DNA) and DNA hydrolases (exonuclease, endonuclease and deoxynucleoside monophosphate kinase) might collaborate with host DNA replication to duplicate the phage genome. DNA breaking-rejoining enzymes and recombination endonuclease VII were important enzymes involved in the process of DNA recombination. Interestingly, hydrogenase was also encoded, and is known to be involved in redox reactions and universally exists in prokaryotes with catalytic activity [9,78]. No sequence homologs to integrases, repressors, transposases or excisionases were identified, further supporting a previous conclusion that AJO1 is a lytic phage. The space between genes is usually occupied by putative regulatory sequences such as promoters and terminators. Eight putative promoters of the AJO1 genome are listed in [Table 2](#), and no putative terminators were found by the FindTerm tool with an energy threshold value of -15 [12].

There were four dispersed repeat sequences (R1–R4) present in this genome (see [Supplementary Table 2](#)), including a long terminal repeat, rolling circle and long interspersed repeat. Several tandem repeats also existed, such as minisatellite DNA and telomeric repeats. These repeat sequences were observed in both coding and intergenic regions. Three of these repeat sequences (R1–R3) occurred on one strand, while the remaining one (R4) occurred on the opposite strand. Since the database of phage genomes is still very limited, the functions of repeats in phages remain unclear. However, based on their functions in host bacteria [1], these genes probably participated in the processes of cis-regulatory elements, epigenetic modification and chromatin reconstruction. No genes associated with pathogenicity/virulence or lysogeny (e.g. integrase encoding) were identified in the genome of AJO1, showing that it might be a good candidate for use in phage therapy and control against *A. johnsonii* infections.

Fig. 3. Rough genome draft of phage AJO1. Sky-blue band indicates gene-encoding regions in the AJO1 genome; orange arrows indicate assembled scaffolds and pink arrows represent predicted ORFs in the AJO1 genome. The GC skew is calculated as $(G - C)/(G + C)$ and the GC plot shows GC% content. The upper vertical line with fixed intervals represents the GC skew (green for positive and purple for negative); the lower vertical lines represent the GC plot (red for above-average and blue for below-average). The upward arrows give the locations of four repeats. Three endonuclease sites and two exonuclease sites are labeled with red and dark blue triangles, respectively.

Table 1
The genome annotation of phage AJO1.

ORF ^a	Coordinates		Protein function (conserved motif) ^b	E value ^c
	Start	End		
1	1	1341	DNA-dependent RNA polymerase (pfam00940)	6.0E–95
2	1335	1664	Unknown	
3	2111	2434	Unknown	
4	2444	3994	Head–tail connecting protein (cl19541)	1.2E–73
5	4007	4747	Putative scaffolding protein (PHA01929)	1.2E–16
6	4766	5776	Capsid protein (cl18086)	2.0E–139
7	5824	6054	Unknown	
8	6185	6754	Tail tubular protein A (cl10205)	6.0E–37
9	6753	9062	Unknown	
10	9064	9765	Unknown	
11	9781	12678	Unknown	
12	12698	15853	Putative internal core protein (cl20117)	6.0E–47
13	15856	16125	Phage T7 tail fiber protein (cl04321)	2.3E–04
14	16130	16822	Unknown	
15	16865	17383	DnaQ-like (or DEDD) 3'–5' exonuclease (cl10012)	4.0E–03
16	17507	18526	Adenylation DNA ligase	2.7E–12
17	18528	19295	DNA endonuclease (COG3780)	2.7E–04
18	19292	20629	RecA-like NTPases (cl17233)	4.0E–06
19	20632	20859	Unknown	
20	21127	21661	Putative DnaG-like primase	9.3E–44
21	21651	21914	Unknown	
22	21914	22057	Unknown	
23	22175	22684	Unknown	
24	22828	23367	Unknown	
25	23378	23689	Unknown	
26	23807	24424	Unknown	
27	24753	25031	Unknown	
28	25031	25174	Hydrogenase (cl19201)	7.0E–06
29	25167	25823	Unknown	
30	25908	26405	Unknown	
31	26413	26622	Unknown	
32	26639	26932	Hypothetical protein (PHA01782)	3.0E–12
33	27133	27590	DNA polymerase (cl02626)	3.0E–03
34	27599	28012	HNH endonuclease (cl02360)	1.6E–07
35	28060	28986	Hypothetical protein (PHA02030)	2.0E–47
36	29070	29393	Unknown	
37	29383	30354	Exonuclease (PHA00439)	5.3E–05
38	30344	30892	Unknown	
39	30885	31334	Recombination endonuclease VII (cl03794)	3.0E–10
40	31318	31509	Unknown	
41	31684	33569	Unknown	
42	33681	34007	Phage lysis protein S (cl04938)	6.0E–05
43	33994	34182	Unknown	
44	34183	36024	Unknown	
45	36021	36140	Unknown	
46	36136	36324	Unknown	
47	36372	36527	Unknown	
48	36953	38169	Phage tail collar domain protein (pfam07484)	8.0E–06
49	38159	38584	Unknown	
50	38629	39267	Deoxynucleoside monophosphate kinase (cl14713)	3.4E–06
51	39272	39664	DNA-directed RNA polymerase (cl20638)	1.0E–05
52	40397	40900	DNA breaking-rejoining enzymes (cl00213)	7.2E–40
53	40935	41252	DUF3596 super family/hypothetical protein (cl12165)	4.7E–03
54	41249	41437	Unknown	

^a ORFs were numbered consecutively.

^b Predicted function was based on amino acid identity, conserved motifs, and gene location within functional modules.

^c The probability of obtaining a match by chance as determined by BLAST analysis. Only values of less than 10^{-3} were considered significant.

3.4. Comparative and phylogenetic analyses

According to comparative analysis results using the tBLASTn algorithm, many AJO1 proteins share high

similarity with homologs of *A. baumannii* phages. At the protein level, 10 *A. baumannii* phages were found with identities of more than 60% (Table 3). In particular, phage vB_AbaP_Acibel007 isolated from clinical sewage [59]

Table 2
Putative promoters of the AJO1 genome.

Start	End	Score ^a	Promoter sequence
7042	7092	0.99	CATCGGAGGATTAAATGGCAGGCAATATGCAGGCAGGTTTATCTACTGG
14789	14839	0.98	TAGAGAATCGTTTAAATAACGCAGGTATTGTTGGGTATACAATCAGAACC
16860	16910	0.97	TCAGACCGCATTAAGTCACCCGTAAGGAAATGTACGAGCGTCGTAAGG
17518	17568	0.98	GCACTGGATGTATATAACAATGAGGACTCTGGAGTGCTTACTGTCATCG
18142	18192	0.99	TGCTCTAGTGCATATATACCCGATGGGCTAGACCCAAAGGACATGCGACC
21756	21806	0.99	AGTCTGCGTCTTTAACTGGGGCGGTTATAAGGCTCAAGAATGTAATCATG
23270	23320	0.97	GGGCAACCTATATTAAGGTTGTCGTGAGGCGAGTTGCCCTGCACTGGCT
26885	26935	0.99	TCGTCGCAGTATTAAGGTGAGCGTAAGCATCGTGAGGCGTTGCTTTGC

^a The score cutoff is 0.97. The transcription start is shown in larger and bolder font.

Table 3
Comparison of phages with BlastN identities of more than 60% against the AJO1 genome.

Phage ^a	Isolated place	Accession	Length (bp)	BlastN score	E value	Query coverage	Identity (%)	Ref.
Acibel007	Belgium	KJ473423.1	42 654	8306	0.0	93	82	[57]
phiAB1	Taiwan	HQ186308.1	41 526	3219	4E–134	61	64	[79]
Abp1	China	JX658790.1	42 185	3260	2E–134	61	64	[80]
AB3	China	KC311669.1	31 185	2307	4E–134	42	64	[81]
phiAB6	Taiwan	KT339321.1	40 570	3210	3E–134	60	64	[82]
IME200	China	KT804908.2	41 243	3270	9E–135	61	64	Submitted (26-JUL-2016)
Petty	USA	KF669656.1	40 739	3183	8E–159	59	78	[83]
PD-AB9	China	KT388103.1	40 938	3258	1E–134	61	64	Submitted (12-AUG-2015)
Fri1	Russia	KR149290.1	41 805	3231	1E–134	59	64	Submitted (18-APR-2015)
PD-6A3	China	KT388102.1	41 563	3253	1E–133	61	64	Submitted (12-AUG-2015)

^a The full name of phages from top to bottom are Acinetobacter phage vB_AbaP_Acibel007, Acinetobacter phage phiAB1, Acinetobacter phage Abp1, Acinetobacter phage AB3, Acinetobacter phage phiAB6, Acinetobacter phage vB_AbaM_IME200, Acinetobacter phage Petty, Acinetobacter phage vB_AbaP_PD-AB9, Acinetobacter phage Fri1, Acinetobacter phage vB_AbaP_PD-6A3.

showed the highest similarity (82%), with the query coverage of more than 90%. Among the 26 matches between both phages, the major structural proteins shared relatively high sequence identities with each other, which explained their similar morphologies. The main differences ranging from 2924 bp to 3556 bp with the lowest identity (52%), were located in the head-tail connecting protein, which might have evolved phage–host adsorption mechanisms [12]. Phylogenetic analysis was also carried out to analyze the relationship among them, and a phylogenetic tree was constructed (Fig. 4). Since phylogenetically related phages are considered to have similar head structural components, they may be clustered according to their major capsid proteins [79]. As expected, phage AJO1 and phage vB_AbaP_Acibel007 are closely clustered, distinguishing them from other *A. baumannii* phages. When the AJO1 genome sequence was compared with the nucleotide database, however, the sequence of phage AJO1 shared only 90% identity with that of phage vB_AbaP_Acibel007, with query coverage of 83%. At the same time, phage vB_AbaP_Acibel007 was reported to be strictly specific towards *A. baumannii*, while phage AJO1 exhibited high host specificity toward *A. johnsonii*. In addition, the optimal MOI, latent period and burst sizes of vB_AbaP_Acibel007 were 10^{–3}, 21 min, 145 phages infected cell^{–1}, respectively, significantly different from those of AJO1 [59]. This finding suggested that the two phages might have descended from a common ancestor, but diverged considerably during evolution.

The above comparative analyses of biological and genetic characteristics suggested that AJO1 is a novel phage belonging to the *Podoviridae* family.

In conclusion, a linear double-stranded DNA phage belonging to the *Podoviridae* family against *A. johnsonii* was isolated from activated sludge in a full-scale municipal wastewater treatment plant during a sludge bulking period. With an icosahedral head 55 ± 2 nm in diameter and a non-contractile tail 8 ± 2 nm in length, it exhibited high host specificity toward *A. johnsonii*, with a burst size of 51.2 phages per infected cell^{–1}. With its relatively rapid growth nature and thermal and pH stabilities, AJO1 might be a very promising candidate as an alternative non-toxic sanitizer for controlling the contamination of *A. johnsonii* in both hospitals and other complicated environments. Genome sequencing revealed that AJO1 was a linear dsDNA (41 437 bp) carrying 54 putative open reading frames and 4 pairs of repeats. The sequence of phage AJO1 shared only 90% identity with that of phage vB_AbaP_Acibel007 with the query coverage of 83%, showing that the newly isolated AJO1 was a novel member of the *Podoviridae* family. No genes evolving pathogenicity/virulence or lysogeny were identified in the genome of AJO1. Since both gene and protein databases of phages are extremely limited, isolation and genome characterization of more phages from various environments are indispensable. We predict that other new phages will be isolated from activated sludge in the near future, thereby contributing to our

Fig. 4. Phylogenetic relationship between phage AJO1 and 10 representatives of *Acinetobacter baumannii* phages. The diagram was constructed by the MEGA7 program using the neighbor-joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches and are in the units of the number of base substitutions per site [80]. The different colors represent different phylogenetic branches, and phages with the same color show a relatively close relationship.

understanding of phage biology and promoting further application.

Conflicts of interest

There is no conflict of interest.

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Appendix A. Supplementary material

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.resmic.2017.01.006>.

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