Biological Characteristics and Genomic Analysis of *Aeromonas Hydrophila* Phage BUCT551 Isolated From Aquatic Sewage

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Abstract

Aeromonas hydrophila is a common opportunistic pathogen in aquaculture and is ubiquitous in aquatic environment. Whereby its accessibility, variety and host specificity, phage is increasingly considered as a promising complementary medicine for antibiotics. However, a small amount of *A. hydrophila* phages have been characterized, which suggests the significance to isolate and characterize novel *A. hydrophila* phages. In this study, we isolated a novel *Aeromonas hydrophila* phage using *A. hydrophila* strain A18 as an indicator and designated it as BUCT551, and it was identified as *Myoviridae* phage by transmission electron microscopy (TEM). The whole genome sequencing of the phage BUCT551 revealed that it has a linear DNA genome of 613,82 bp. BLASTn analysis showed that phage BUCT551 shared 86.75% homology with *A. hydrophila* phage LAh_7 (Genbank ID: MK838113.1). The one-step growth curve demonstrated that phage BUCT551 had a latent period of 20 min and the burst size of 32 pfu/cell at its optimal MOI of 0.1. The phage BUCT551 had a survival pH range from 5 to 10 and tolerant temperature from 0°C to 40°C. Host range analysis shown that the phage was able to lyse not only *A. hydrophila*, but also *A. veronii*.

Introduction

Aeromonas hydrophila is a gram-negative, non-spore forming, rod-shaped and facultative anaerobic organism belonging to genus *Aeromonas* family *Aeromonadaceae* [1]. It is ubiquitous in natural environment because of its wide range of survival and reproductive temperature from 4°C to 40°C, usually found in aquatic environment like river fresh water, and it is recognized as an opportunistic pathogenic of poikilothermic and homoeothermic animals including human [2, 3]. *A. hydrophila* is regarded as a causative agents pathogen of many fish species involved in multiple diseases, including ulcerative disease, hemorrhagic disease, red sore disease, and septicemia in fish, which leads to the severe economic loss to the aquaculture industry [4–6]. In addition, *A. hydrophila* is pathogenic to human especially immunocompromised people by means of colonizing in human intestinal tract, and it is linked to gastroenteritis, skin diseases and septicemia [4, 6, 7, 8–10]. *A. hydrophila* produces virulence factors including aerolysin, hemolysin, adhesion factor, enterotoxin and extracellular protease that results in tissue damage, which act individually or in synergy, contributing to the pathogenicity of *A. hydrophila* [8]. *Aeromonas* infections have been reported in recent decades, and a statistic from Japan indicated *Aeromonas* species were isolated from 5% travelers (23,215 persons in total) who returned from the developing countries [9]. In 2018 to 2019, Hilt et al. isolated several multi-drug resistant *A. hydrophila* isolates from two solid organ transplant patients [11]. In another case, Hasan et al. recently reported the *Aeromonas hydrophila* infection in an immunocompromised 69 years old female, with surgical site sepsis/infection and several comorbidities [12].

The bacteria antibiotics resistance is a great challenge and threat to the human life [13, 14], and antibiotics resistance of aeromonas has attracted more and more attention in recent years. Elbehiry et al. isolated *A. hydrophila* from 150 meat and water samples and to screen their antibiotics resistance, and found that some *A. hydrophila* strains were resistant to ampicillin, cefotaxime, pefloxacin, ceftazidime and ciprofloxacin [15]. In another study, *A. hydrophila* was detected to carry an acquired gene encoding the carbapenemase GES-24, indicating its property of carbapenem-resistant [16]. Given that *A. hydrophila* exhibits pathogenicity to both humans and animals, and its outbreak may lead to economic loss in agriculture and aquaculture or public health incident, the potential antibiotic resistance of *A. hydrophila* should be paid to the attention.

Under the circumstance of antibiotic drug resistance, phage therapy was found more and more important in the antibacterial theory either using alone or in combination with antibiotics [17–19]. Phage is exclusively virulent to prokaryotic microbe and do not lyse animal and human cells. Phages are classified into lytic phage and lysogenic phage depending on their life cycle. Lytic phage lyses host bacteria immediately after accomplishing replication and assembly process in the host, consequently leading to the elimination of host bacteria. The characteristic of lytic phage endows therapeutic potential of phage as a treatment for bacterial infection [10]. Some phages demonstrate high specificity to a single species or even a certain strain of bacteria [20]. To enhance the efficacy when using phage as antibacterial agents, a strategy that formulate phages into cocktails comes out [6]. However, only few *A. hydrophila* phages are isolated and characterized [21]. Here our study described a novel *A. hydrophila* phage with 86.75% genome coverage to unpublished *A. hydrophila* phage LAh_7, and found that the phage could lyse *aeromonas veronii*. Our study provides a new option for the treatment of *aeromonas* infections.

Materials And Methods

Bacterial Strain Isolation and Identification

The host strain A18 was isolated from sewage water of aquatic product market (Nanjing, China). The molecular identification of A18 was performed through sequence analysis of 16S rRNA gene. Microbial DNA was extracted and purified from 1 mL individual bacterial colonies culture using extraction kit according to manufacture's instructions. Subsequently, the purified DNA was applied to amplify the V3-V4 region of the 16S rRNA gene using primers reported previously [23]. According to the method described by Pan [23], we confirmed that A18 belonged to species *Aeromonas hydrophila*. The strain A18 was cultured at 28 ~ 30°C and 220×g in Tryptic Soy Broth (TSB) medium (BD Difco) [23].

Phage isolation and purification

The sewage water sample collected from Nanjing aquatic market was centrifugated at 12000×g for 10 min, followed by supernatant filtration using 0.22 µm membrane. Phage filtrate was pipetted into culture solution with *A. hydrophila* strain A18 of logarithmic phase. The mixture was incubated overnight at 28°C in the shaker to allow phages to propagate in a large scale, and was centrifuged at 12000×g for 3 min to get rid of bacterial cells and debris. In order to further remove residual cells and debris, the supernatant was filtered by 0.22 µm membrane. The phage stock was obtained after centrifugation and filtration. Verification of phage was warranted to pipetted phage stock onto double-layer agar plates with the upper agar mixing with A18 and incubated until the appearance of plaque. Phage isolate purification was performed subsequently when the phage was verified. After serial 10-fold dilution of the stock solution, diluents with different concentrations were mixed with host bacteria, and the mixture was added to TSB medium to make double-layer agar plates. After incubation, single plaque was selected and cultured in TSB medium using A18 as an indicator. Phages were re-harvested by centrifugation and filtration. The purification processes were conducted for three times [24].

Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) was performed to determine the morphological characteristics of phage after concentration using cesium chloride (CsCl) gradient centrifugation [25]. 20 µL of purified phages suspension was pipetted onto carbon-coated support membrane copper mesh and retained 20 min, followed by negative staining with 2% phosphotungstic acid solution for 1 min retention. After further drying, phage particles were observed under an electron microscope (JEM-1200EX, Japan) at an accelerating voltage of 100 kV.

Phage genomic DNA extraction

The genome of the isolated phage was extracted for sequencing. According to the method described by Tang et al. and with slight modification, genomic DNA of the phage was extracted by proteinase K /SDS method [26]. DNase I and RNase A (Thermo Scientific, USA) were introduced to purified phage to the final concentration of 1 mg/L, and was incubated overnight at 37 °C. Then the solution was heated at 80 °C for 15 min for enzyme inactivation. EDTA, proteinase K and SDS were added to final concentration of 0.02 mol/L, 50 mg/L and 0.5%, respectively, and the solution was incubated at 56 °C for 1 h. After incubation, balanced phenol was added at the same volume and the mixture was centrifuged at 12000×g for 10 min. The supernatant was collected and the same volume of phenol/chloroform/isoamyl alcohol (25:24:1, by volume) was added, then centrifuged at 12000×g for 10 min. Mixed the supernatant with 400 µL of isoamyl alcohol (Sinopharm Chemical Reagent Co., Ltd.) and retained at -20 °C for more than 1 h. The mixture was centrifuged at 12000×g for 20 min at 4°C, then the liquid was drained to harvest the precipitation. The precipitation was washed with 75% pre-cooled ethanol (-20°C) and centrifuged at 12000×g for 10 min at 4°C. Removed the ethanol and collected the the DNA precipitation, the precipitation was resuspended in nucleic acid-free water.

Determination of the Multiplicity of Infection (MOI)

Multiplicity of infection (MOI) signifies the ratio of the number phages in a system to the total amount of bacteria and it can be interpreted as the number of phages added to each cell during the infection. Dilute phage stock solution and bacterial suspension in 10-fold serially to various concentrations, mixed then proportionally to acquire infectious systems with different MOI. Phage titers were determined by double layer agar method after incubated overnight at 28°C [27].

Thermal Stability and pH Sensitivity

In order to investigate the vigor of phage BUCT551 in multiple environmental condition, we measured the phage titers in diverse temperatures and values of pH with the method previously described [27]. In thermal stability assay, phages (approximately 10^9 pfu/mL) were incubated in water bath at different temperatures (4°C, 20°C, 30°C, 40°C, 50°C, 60°C, 70°C) for an hour, and then the phage titers were determined. Similarly, phages were incubated at 28°C in the TSB medium with different pH values for an hour, then phage titers were calculated [3]. All experiments were performed in triplicate and results were averaged.

One-Step Growth Curve Assay

The one-step growth curve assay was implemented with the purpose of determining the eclipse period and burst size of phage BUCT551. The phage BUCT551 was introduced to *A. hydrophila* strain A18 in exponential phase at the optimal MOI and was allowed for 30 min absorption at 28°C. Mixture was centrifugated at 12000×g for 1 min to separate free phage particles with host bacteria immediately after the absorption procedure. Resuspended the sediment and cultured at 28°C in the shaker for 205 min and samples were collected at 0, 10, 20, 30, 40, 55, 70, 85, 100, 115, 130, 145, 160, 175, 190 and 205 min during incubation, and phage titers were determined by double-layer agar method as described above. Taking the infection time (minute) as the abscissa and the phage titer (pfu/mL) as the ordinate, and the one-step growth curve was drawn, clearly the eclipse and lysis periods of the phage were obtained, and the amount of phage released was calculated. The assay was conducted in triplicate and results were averaged [28].

Host range analysis

The bacterial susceptibility level was inspected using spotting methods as previously described [24]. About 20 strains of *Vibrio Parahemolyticus* that were stored in Biosafety Technology Research Center, Beijing University of Chemical Technology and 4 strains of *Aeromonas hydrophila* were selected randomly for host range determination. Transferred 500 µL of the *A. hydrophila* culture to 5 mL of TSB medium with 0.75 % agar and promptly poured on a plate paved with tryptic soy agar (TSA). After solidification, pipetted the phage BUCT551 onto double-layer agar, then the plates were cultured overnight to observe the plaque formation.

Drug sensitivity test of host strain A18

In order to further prevent the contamination caused by *Aeromonas*, we tested the sensibility of A18 to some common antibiotics. Drug sensitivity test was performed using inhibition zone method on the grounds of "Antimicrobial Sensitivity Test Standard". 12 kinds of common antibiotics in clinic were involved in the test. Bacterial suspension was evenly spreaded in the plate, then various disks with antibiotics were placed on the plates to allow for 24 h incubation at 28°C, the diameter of the inhibition zone was measured for drug sensitivity evaluation [29].

Whole-genome Sequencing Analysis and Bioinformatic Analysis

The phage BUCT551 DNA library was constructed in accordance to NEBNext Fast DNA Library Prep Set for Ion Torrent protocol kit. The concentration of nucleic acid was determined by Qubit 2.0 (Invitrogen, USA). Phage whole-genome sequencing for the phage BUCT551 was performed using MiSeq (Illumina, USA) platform. Newbler V3.0 software (Roche 454) and CLC software (CLC Bio) were used to genomic sequence assembling [30]. Low-quality sequences were filtered out using the Trimmomatic (V0.32) program.

Phylogenetic tree was constructed based on the amino acid sequene of conserved major capsid protein in order to analyze the relationship between the new isolate phage BUCT551 and other phages of the same subfamily recorded by the International Committee on Taxonomy of Viruses database (ICTV). MEGA version 7.0 was employed to constructed phylogenetic tree.

Results And Discussion

Phage Morphology

Aeromonas hydrophila phage was isolated from aquacultural waste water and was named as BUCT551. Transmission electron microscope revealed that the phage BUCT551 had a polyhedral capsid(50 ± 3 nm in diameter)and a long tail 180 ± 5 nm(Fig. 1). Baesd on the latest ICTV classification system, it belonged to the family *Myoviridae*.

Thermal Stability and pH Sensitivity

The thermal stability assay showed that the phage BUCT551 demonstrated vitality from the temperature range of 4°C~40°C. The survival rate decreased significantly over 50°C, and the phage was inactivated completely after incubating at 70°C for 60 min (Fig. 2a). The pH sensitivity test showed that the phage exhibited good vitality in the pH range of 5 ~ 8. Viral titers drastically dropped when the phage BUCT551 was incubated for an hour at the pH of 4 and 11, indicating that the growth of the phage BUCT551 was inhibited in extreme pH environment (Fig. 2b).

Optimal MOI and One-Step Growth Curve

The virion productively of the phage BUCT551 using different MOI fluctuated slightly. Based on the maximum titer obtained from phage BUCT551 infection of *A. hydrophila* strain A18, the optimal MOI of the phage BUCT551 was 0.1 (Fig. 3a). One-step growth curve assay exhibited that the phage BUCT551 had a latent period of 20 minutes and a growth period of 85 minutes, and reached the plateau at the 105 minutes, when the viral titer was 10^9 pfu/mL. The burst size of the phage BUCT551 was 32 pfu/cell (Fig. 3b).

Host Range analysis

In order to identify the host strain of BUCT551, all the 9 strains of *A. hydrophila* (Ah 154, ATCC 419140, AS1. 18031, Ah 7, Ah 9, Ah 10, Ah 17, Ah 18, No. 201308071) and 8 strains of *A. veronii* (Av 4, Av 6, Av 15, Av 26, Av 25, Av 13, Av 21, Av 3) in our lab and 20 strains of *vibrio parahaemolyticus* (for control) were selected as candidate hosts for the reason that *A. hydrophila* and *vibrio parahaemolyticus* shared an identical family *vibrionaceae*. Result exhibited that 1 strain of *A. hydrophila* and 4 strains of *A. veronii* were susceptible to the phage BUCT551, suggesting that BUCT551 was potential to be employed as a treatment to *Aeromonas* infection (Table 1).

Table 1
Host range analysis of the phage BUCT551

Bacteria	Genus, species	susceptibility
Av 3	<i>Aeromonas, veronii</i>	susceptible
Av 4	<i>Aeromonas, veronii</i>	not susceptible
Av 6	<i>Aeromonas, veronii</i>	not susceptible
Av 13	<i>Aeromonas, veronii</i>	not susceptible
Av 15	<i>Aeromonas, veronii</i>	not susceptible
Av 21	<i>Aeromonas, veronii</i>	susceptible
Av 25	<i>Aeromonas, veronii</i>	susceptible
Av 26	<i>Aeromonas, veronii</i>	susceptible
Ah 7	<i>Aeromonas, hydrophila</i>	susceptible
Ah 9	<i>Aeromonas, hydrophila</i>	not susceptible
Ah 10	<i>Aeromonas, hydrophila</i>	not susceptible
Ah 17	<i>Aeromonas, hydrophila</i>	not susceptible
Ah 18	<i>Aeromonas, hydrophila</i>	not susceptible
No.2013080717	<i>Aeromonas, hydrophila</i>	not susceptible
ATCC 419140	<i>Aeromonas, hydrophila</i>	not susceptible
AS1.18031,	<i>Aeromonas, hydrophila</i>	not susceptible
Results of <i>Vibrio parahaemolyticus</i> were not shown in the Table 1.		

Drug sensitivity analysis of *Aeromonas hydrophila* A18

The host bacteria A18 was highly sensitive to cefalexin, ceftriaxone, cefalotin, cefaclor, ofloxacin and norfloxacin while developed resistance to erythromycin, penicillin, roxithromycin and amoxicillin (Table 2).

Table 2
Sensibility of *Aeromonas hydrophila* strain A18 to twelve kinds of antibiotics

Antibiotics	Zone of inhibition (mm)	Sensibility
cefalexin	28	S
erythromycin	< 10	R
penicillin	< 10	R
roxithromycin	< 10	R
amoxicillin	< 10	R
ceftriaxone	45	S
cefazolin	11	I
ofloxacin	28	S
cephalothin	26	S
tetracycline	18	I
cefaclor	30	S
fleroxacin	24	S

Whole-genome analysis

The whole genome sequencing revealed that BUCT551 had a linear genome of 61,382 bp with the C + G content of 61.77% (accession number MT952005) (Fig. 4). According to the homology comparison of the whole genome sequence, we found that BUCT551 exhibited the highest homology with *Aeromonas* phage LAh_7 (Fig. 5). Based on the peculiarity that restriction endonucleases specifically function on double-strand DNA, restriction endonucleases digestion then electrophoresis were able to determine the characteristic of nucleic acid. The phage BUCT551 genome enzyme-digested product exhibited multiple electrophoretic bands, which preliminary verified that BUCT551 belonged to double-stranded DNA phage (Fig. 6).

Functional ORFs analysis

BLASTn analysis indicated that BUCT551 shared 86.75% homology (87% genome coverage) with unpublished *Aeromonas hydrophila* phage LAh_7. RAST gene annotation identified that the phage genome contained 74 open reading frames (ORFs), none of which corresponded to tRNA gene, among which ATG was the initiation codon of most ORFs. Among the predicted protein coding genes, 27 of the 74 ORFs were identified as functional coding sequences, and the rest were annotated as functional unknown proteins or hypothetical proteins (Table 3).

Table 3
Predicted ORFs in the genome of phage BUCT551

ORFs	Start	Stop	Function	Best-match BLASTp Result	Query Cover	E-values	Gen Bank Acc. No
ORF1	210	866	hypothetical protein	HNH endonuclease	100%	4e-112	QFG04404.1
ORF2	2902	920	hypothetical protein	hypothetical protein	76%	0.0	QDH46647.1
ORF3	3915	2986	hypothetical protein	hypothetical protein	99%	3e-166	QFG04406.1
ORF4	4721	4044	hypothetical protein	hypothetical protein LAh7_4	100%	3e-142	QDH46667.1
ORF5	4921	4721	hypothetical protein	hypothetical protein LAh7_5	100%	2e-35	QDH46713.1
ORF6	5979	4918	hypothetical protein	hypothetical protein LAh7_6	100%	7e-172	QDH46657.1
ORF7	6379	6047	hypothetical protein	hypothetical protein	98%	6e-63	QFG04410.1
ORF8	6656	6381	hypothetical protein	hypothetical protein	100%	5e-43	QFG04411.1
ORF9	7096	6653	hypothetical protein	hypothetical protein LAh7_9	98%	1e-75	QDH46683.1
ORF10	7249	7100	hypothetical protein	hypothetical protein	100%	8e-08	QFG04413.1
ORF11	7584	7249	hypothetical protein	hypothetical protein LAh7_11	100%	2e-66	QDH46694.1
ORF12	8051	7587	hypothetical protein	hypothetical protein LAh7_12	100%	9e-88	QDH46680.1
ORF13	8329	8048	hypothetical protein	hypothetical protein LAh7_13	83%	2e-25	QDH46701.1
ORF14	8663	8475	hypothetical protein	hypothetical protein LAh7_14	93%	7e-09	QDH46710.1
ORF15	9189	8656	Phage protein	hypothetical protein LAh7_15	100%	2e-95	QDH46675.1
ORF16	9859	9266	hypothetical protein	hypothetical protein	100%	6e-129	QFG04419.1
ORF17	10466	9870	Adenylate cyclase (EC 4.6.1.1)	hypothetical protein LAh7_17	100%	1e-114	QDH46672.1
ORF18	10591	11244	hypothetical protein	DNRLRE domain-containing protein	81%	5e-25	TPV95153.1
ORF19	12302	11334	hypothetical protein	hypothetical protein LAh7_19	100%	2e-135	QDH46659.1
ORF20	12687	12313	hypothetical protein	GntR family transcriptional regulator	100%	2e-28	QFG04422.1
ORF21	13093	12677	hypothetical protein	hypothetical protein LAh7_20	100%	6e-79	QDH46687.1
ORF22	13516	13265	hypothetical protein	hypothetical protein LAh7_21	100%	9e-54	QDH46706.1

ORFs	Start	Stop	Function	Best-match BLASTp Result	Query Cover	E-values	Gen Bank Acc. No
ORF23	13713	14054	hypothetical protein	hypothetical protein LAh7_23	96%	1e-54	QDH46695.1
ORF24	14074	14730	hypothetical protein	hypothetical protein	100%	6e-122	QFG04426.1
ORF25	14727	14942	hypothetical protein	RES domain-containing protein	100%	4e-37	QFG04427.1
ORF26	14942	15403	hypothetical protein	hypothetical protein LAh7_26	100%	2e-89	QDH46679.1
ORF27	15400	16074	hypothetical protein	hypothetical protein LAh7_28	99%	4e-133	QDH46668.1
ORF28	16085	17647	hypothetical protein	exonuclease	100%	0.0	QFG04431.1
ORF29	17631	17879	hypothetical protein	hypothetical protein LAh7_30	95%	2e-49	QDH46699.1
ORF30	18022	18378	hypothetical protein	hypothetical protein LAh7_32	100%	3e-68	QDH46691.1
ORF31	18375	18671	hypothetical protein	hypothetical protein	100%	2e-18	QFG04434.1
ORF32	18668	19348	hypothetical protein	putative 3'-5' exoribonuclease	100%	4e-142	QDH46666.1
ORF33	19360	19782	hypothetical protein	hypothetical protein LAh7_35	100%	9e-96	QDH46684.1
ORF34	19784	20581	hypothetical protein	putative 3'-5' exoribonuclease	100%	2e-180	QDH46660.1
ORF35	20595	21356	putative DNA adenine methylase	putative N-6-adenine-methyltransferase	98%	5e-173	QDH46661.1
ORF36	21356	21793	hypothetical protein	hypothetical protein LAh7_38	100%	1e-73	QDH46686.1
ORF37	21894	23342	hypothetical protein	putativeDNAligase/BRCA1domainprotein	100%	0.0	QDH46651.1
ORF38	23372	23566	hypothetical protein	hypothetical protein	81%	2e-09	QFG04441.1
ORF39	23610	24257	hypothetical protein	putative 3'-phosphatase, 5'-polynucleotide kinase	100%	1e-137	QDH46665.1
ORF40	24267	24659	hypothetical protein	hypothetical protein LAh7_42	99%	1e-81	QDH46690.1
ORF41	24727	24852	hypothetical protein	NO FOUND	100%	0.0	QAY02159.1
ORF42	24855	25346	Permease of the drug/metabolite transporter (DMT) superfamily	hypothetical protein LAh7_43	100%	1e-109	QDH46678.1
ORF43	25343	25594	hypothetical protein	hypothetical protein	100%	1e-36	QFG04446.1

ORFs	Start	Stop	Function	Best-match BLASTp Result	Query Cover	E-values	Gen Bank Acc. No
ORF44	26010	25762	hypothetical protein	hypothetical protein LAh7_46	100%	4e-29	QDH46703.1
ORF45	26714	26007	hypothetical protein	putative lysis protein A	97%	2e-155	QDH46662.1
ORF46	28129	26768	hypothetical protein	hypothetical protein	46%	0.40	QFG04449.1
ORF47	32540	28224	hypothetical protein	putative tail protein	63%	0.0	QDH46642.1
ORF48	32952	32728	hypothetical protein	hypothetical protein LAh7_51	100%	6e-44	QDH46711.1
ORF49	33783	32962	hypothetical protein	DUF2163 domain-containing protein	100%	5e-138	QFG04452.1
ORF50	34973	33780	hypothetical protein	hypothetical protein LAh7_53	100%	0.0	QDH46654.1
ORF51	39412	34973	hypothetical protein	putative tape measure protein	97%	0.0	QDH46643.1
ORF52	39518	39405	hypothetical protein	hypothetical protein	80%	1e-08	QFG04455.1
ORF53	40111	39662	hypothetical protein	putative tail assembly chaperone	99%	1e-77	QDH46682.1
ORF54	41414	40269	hypothetical protein	putative major tail protein	100%	0.0	QDH46656.1
ORF55	41953	41417	hypothetical protein	putative minor tail protein	99%	2e-102	QDH46673.1
ORF56	42557	41946	Phage tail, component Z	hypothetical protein LAh7_59	99%	4e-139	QDH46670.1
ORF57	42904	42554	hypothetical protein	hypothetical protein	100%	5e-60	QFG04460.1
ORF58	43315	42908	hypothetical protein	hypothetical protein	100%	2e-35	QFG04461.1
ORF59	44412	43372	Phage capsid and scaffold	putative major capsid protein	100%	0.0	QDH46658.1
ORF60	44817	44428	hypothetical protein	decorator protein	99%	1e-75	QFG04463.1
ORF61	46103	44820	Phage head, head-tail preconnector protease C / Phage head, scaffolding domain Nu3	putative S49 family peptidase	100%	0.0	QDH46655.1
ORF62	47815	46100	Phage head, portal protein B	putative portal protein	100%	0.0	QDH46648.1
ORF63	48054	47815	hypothetical protein	hypothetical protein LAh7_65	100%	1e-50	QDH46708.1

ORFs	Start	Stop	Function	Best-match BLASTp Result	Query Cover	E-values	Gen Bank Acc. No
ORF64	50153	48051	Phage head, terminase DNA packaging protein A	putative terminase large subunit	100%	0.0	QDH46646.1
ORF65	50718	50119	hypothetical protein	putative terminase small subunit	99%	4e-135	QDH46671.1
ORF66	52175	50727	hypothetical protein	putative ATP-dependent helicase	99%	0.0	QDH46649.1
ORF67	52512	52222	hypothetical protein	hypothetical protein LAh7_69	100%	6e-60	QDH46696.1
ORF68	54622	52514	DNA polymerase I	putative DNA polymerase	100%	0.0	QDH46645.1
ORF69	55312	54683	hypothetical protein	hypothetical protein LAh7_71	100%	9e-141	QDH46669.1
ORF70	56772	55363	Phage protein (ACLAME 116)	DUF2800 domain-containing protein	100%	0.0	QFG04472.1
ORF71	57128	56772	hypothetical protein	hypothetical protein LAh7_73	81%	8e-48	QDH46688.1
ORF72	57385	57654	hypothetical protein	hypothetical protein	94%	1e-39	QFG04474.1
ORF73	60365	57684	hypothetical protein	hypothetical protein	100%	0.0	QFG04475.1
ORF74	60626	60384	hypothetical protein	putative AlpA family regulatory protein	100%	2e-48	QDH46707.1

We classified the 27 proteins with known functions into four functional divisions: replication division, modification and regulation division, structural and packaging division and lytic division. Both BLASTp and RAST analysis demonstrated that the ORF64 and ORF65 encoded the large subunit and small subunit of terminase, respectively, which played a significant role in phage DNA packaging. With regard to the phage replication division, ORF68 (DNA polymerase), ORF32 (putative 3'-5' exoribonuclease), ORF34 (putative 3'-5' exoribonuclease), and ORF39 (putative 3'-phosphatase, 5'-polynucleotide kinase) encoded by phage BUCT551 participated in the regulation of phage DNA replication. In addition, DNA helicase encoded by ORF66 and HNH endonuclease by ORF1 were speculated to play a crucial role in DNA replication and regulation, and the subsequent modification [31]. ORF35 (N-6-adenine-methyltransferase) was likely to participate in phage DNA modification, whose aim was to circumvent the protection system of host bacteria [32].

Phage structural proteins were encoded by multiple ORFs including ORF47 (putative tail protein), ORF51 (putative tape measure protein), ORF54 (putative major tail protein), ORF55 (putative minor tail protein), ORF53 (putative tail assembly chaperone), ORF59 (putative major capsid protein) and ORF62 (putative portal protein). Portal protein encoded by ORF62 was considered to function in the channel formation of capsid and phage genome injection into host cells [33]. The protein encoded by ORF51 could assist and regulate phage tail assembly and was identified as an essential protein that assisted phage attachment to host cells during infection period [34].

ORF18 (DNRLRE domain-containing protein), ORF20 (GntR family transcriptional regulator), ORF25 (RES domain-containing protein), ORF49 (DUF2163 domain-containing protein), ORF53 (putative tail assembly chaperone), ORF60 (decorator protein) and ORF61 (putative S49 family peptidase) encoded proteins that were considered to play roles in phage modification and regulation. ORF53 encoding tail assembly chaperone widely existed in the genomes of *Siphoviridae* and *Myococcal* phages, and generally located between tail protein and tape measure protein, participating in the regulation of frameshift [35]. ORF60 encoded decorator proteins, a class of proteins that located on the surface of certain phage capsids especially the phages with double-strand DNA,

which able to protect the phage from extreme changes such as environmental changes by enhancing and promoting the robustness of the capsid. Moreover, many decorator proteins possessed other function, such as facilitating target cell recognition, involving in phage assembling or regulating host genes expression, etc. Previous researches had demonstrated that GntR family of transcription factors encoded by ORF20 generally existed in prokaryotes and certain virus, which regulated various kinds of important metabolism pathways. GntR family of transcription factors were recognized to play an irreplaceable role in the growth and reproduction in prokaryotes, and were inferred to protect phages from extreme temperature and pH, etc [36].

ORF45 (putative lysis protein A) and ORF74 (putative AlpA family regulatory protein) belonged to phage lytic division, they probably encoded lytic peptidases and enabled phage to lyse host cells effectively [37].

It was worth noting that 47 ORFs were classified as functional unknown proteins, which accounted for the majority of the overall ORFs. Future researches are warranted to further analyze proteins encoded by ORFs of unknown function so as to gain an insight into phages.

Phylogenetic tree analysis for phage BUCT551

The evolutionary relationship between the phage BUCT551 and other phages was determined on the basis of the sequence of a conserved major capsid protein (ORF59) to determine the phylogeny of BUCT551, which was commonly used as a marker gene and to study phage diversity [38, 39]. Phylogenetic analysis revealed that BUCT551 (MT952005.1) was mostly related to the phage LAh_7 (MK838113.1), which belonged to the family *Siphoviridae* (Fig. 7).

Discussion

Aeromonas hydrophila is considered as an opportunistic pathogenic bacteria, infecting dozens of cultured fishes. *Aeromonas hydrophila* are pathogenic to fishes such as Atlantic salmon, trout, turbot, etc, and the cardinal symptoms of *A. hydrophila* infection in fish are tail bleeding and the epidermis fester [4]. At present, the main prevention and treatment of *A. hydrophila* infection still depend on disinfectants and antibiotics. However, the long-term use of antibiotics may lead to drug resistance. Given this inevitable situation, novel antibacterial therapies are deserved to be researched and exploited. As a novel type of antibacterial agent, phage possesses a series of strengths such as no obvious side effects, easily acquired, and no residue. Phage therapy provides a new alternative to control diseases caused by *Aeromonas hydrophila*.

BUCT551 had a double-strand DNA genome with the length of 61,382 bp, and the C + G content was 61.77%. In the genome of the phage BUCT551, we found no genes (such as gene encoding integrase) were associated with the viral genome integration (Table 2). Through the predicted protein, we found that the phage BUCT551 encoded N-6-adenine-methyltransferase (ORF35), which played a vital role in escaping the protective system of bacteria. In addition, in the analysis of other proteins that may be expressed by phages, we did not identify any genes related to toxins or virulence factors, which indicated that phage BUCT551 was promising to use as a therapeutic agent for *Aeromonas hydrophila* A18.

In this study, we successfully isolated a novel phage BUCT551 against *Aeromonas hydrophila* from sewage samples in an aquatic products wholesale market. Morphological and genetic characteristics indicated that the phage BUCT551 belonged to *Myoviridae*. The one-step growth curve of the phage BUCT551 at the optimal multiplicity of infection (MOI) showed that it possessed characteristics of short incubation period and fast growth rate, and further confirmed that the phage BUCT551 was highly lytic. The phage BUCT551 showed a good viability below 40°C, indicating that it has the similar growth temperature to its host, and pH stability test shown that phage activity remained relatively stable in the pH range from 5 to 10. In conclusion, the above phage biological characteristics indicated that the phage BUCT551 had robust and stable bacteriolytic competence, which is an important basis for phage therapy. In the host range analysis assay, we detected the susceptibility of 16 strains of *aeromonas*, and found that 5 of them were susceptible to the phage BUCT551. In view of the small amount of *A. hydrophila* phages that have been isolated and characterized, the phage BUCT551 has the potential to remedy the *A. hydrophila* and *A. veronii* infection.

Conclusions

In conclusion, due to the large-scale use of antibiotics, the application of phage to control bacterial infection of fish in the aquatic environment exhibited more sustainability and security. In this study, the *Aeromonas hydrophila* phage BUCT551 had been identified and characterized, and results indicated that the phage BUCT551, along with other *A. hydrophila* phages, had a great potential to use in combination with antibiotics. Besides, given the lack of identified *Aeromonas hydrophila* phages, exploring the large amounts of hypothetical proteins encoded in its genome will be the priority of our future research.

Declarations

Author Contributions

Hongbo Qin and Shiting He drafted the manuscript and carried out experiments. Hongbo Qin, Shiting He, Ke Liu, Fuxing Lou, Shuqi Wang and Xiaoping An analyzed the data, Lihua Song, Huahao Fan and Yigang Tong designed the experiment and revised the manuscript.

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Conflicts of Interest

All the authors declare no competing interests.

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Figures

Figure 1

The morphology of phage BUCT55 as seen in an transmission electron microscope (TEM).

Figure 2

a. The survival rate decreased drastically when treated with temperature over 30°C. b. The survival rates maintain stability with pH from 5 to 8.

Figure 3

a. Result of phage titers measured in different multiplicity of infection (MOI) showed the optimal MOI was 0.1. b. Phage titers at different time of post infection.

Figure 4

The homology comparison between BUCT551 and its proximal phages. The gradual red vertical bar in the lower right corner of the figure indicates the genetic homology between the phage BUCT551 and the other phage in the picture.

Figure 5

BUCT551 whole genome digested by restriction enzyme EcoR $\square$, BamH $\square$ and Hind $\square$.

Figure 6

Whole genome map of BUCT551. The outermost layer displayed 74 open reading frames encoded in the genome, different colors represent different functions. The innermost circle represents the GC skew (G-C/G+C. Outwards indicates > 0 and inwards indicates < 0)

Figure 7

Phylogenetic analysis of BUCT551. An evolutionary tree was constructed using capsid proteins. Phylogenetic trees were constructed using the neighbor joining method of 1000 bootstrap copies in MEGA7.