Characterization and genome analysis of two novel *Aeromonas hydrophila* phages PZL-Ah1and PZL-Ah8

Huabo Yu
Jilin Agricultural University
Chao Feng
Jilin Agricultural University
Liang Zhang
Jilin Agricultural University
Teng Chi
Jilin Agricultural University
Yanling Qi
Jilin Agricultural University
Kaixiang Jia
Jilin Agricultural University
Yang Zhang
Jilin Agricultural University
Junfeng Wei
Jilin Agricultural University
Aidong Qian
Jilin Agricultural University
Wuwen Sun
Jilin Agricultural University
Xiaofeng Shan
Jilin Agricultural University
Lei Zhang (zhanglei0221@jlau.edu.cn)
Jilin Agricultural University https://orcid.org/0000-0002-1869-5096

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Abstract

Aeromonas hydrophila (*A.hydrophila*) is an opportunistic pathogen of fish-human-livestock, which poses a seriously affects to the development of aquaculture. Phage therapy is considered as a process to alternatively control bacterial infections and contaminations. In this study, the genomes of two *Aeromonas hydrophila*- specific phages PZL-Ah1 and PZL-Ah8 were isolated, characterized and genomic sequence analyzed. Transmission electron microscopy showed that the two phages had been classified as the member of the *Podoviridae* family. Both the two phages in this study had relatively narrow host range with lytic activity against *Aeromonas spp.* strains. However, they could lyse 3 common *A.hydrophila* strain. As revealed from the whole genomic sequence analysis, PZL-Ah1 and PZL-Ah8 coverd the double-stranded genome of 38,641 bp and 40,855 bp in length, with the GC content of 53.68% and 51.89%, respectively. Through gene comparison in NCBI database revealed that PZL-Ah1 and PZL-Ah8 were 97.67% – 95.51% identical to *Stenotrophomonas* phage IME15 and *Aeromonas* Phage T7-Ah. Phylogenetic analysis showed that PZL-Ah8, PZL-Ah1 and other two phages belonged to the same genus. A total of 44 and 52 open reading frames (ORFs) were predicted in the PZL-Ah1 and PZL-Ah8 genome, respectively. In the process of gene annotation, 28 (63.6%) ORFs in PZL-Ah1 and 29 (55.8%) ORFs in PZL-Ah8 were known to functional proteins in NCBI database, while the remaining ORFs were classified as “hypothetical proteins”, whose functions were yet unknown. By comparing, ORF 02, ORF 29 and ORF 04 in PZL-Ah1, ORF24 in PZL-Ah8 were responsible for the host cell lysis. In conclusion, genomic studies of these two novel phages would lay the foundation for expanding the phage genome database and providing good candidates for phage typing applications.

1. Introduction

Aeromonas hydrophila, pertaining to the genus *Aeromonas* of *Vibrio* family, refers to a type of gram-negative short *Bacilli*. Over the past few years, it had imposed significant economic losses to aquaculture industry, thereby caused fish, frogs and soft-shelled turtles to bacterial septicemia or hemorrhagic disease [1]. Moreover, *A.hydrophila*, as a fish-human-livestock symbiotic bacterium, it could also cause septicemia and acute gastroenteritis in humans [2, 3]. The existing treatment of *A.hydrophila* was the dependence of antibiotics as well as some chemical drugs. In contrast, continued use of antibiotics could lead to the development of resistant bacteria. The problem of bacterial drug resistance was increasing seriously [4, 5]. Thus, novel drugs capable of effectively treating the infection attributed by resistant *A.hydrophila* should be urgently developed.

Bacteriophage as a bacterial virus, could lyse and kill bacteria. Bacteriophage therapy exhibited significant advantages over antibiotics when treated bacterial infection. It was capable of killing bacteria specifically with lower cost and easy-to-use characteristics [6, 7]. As a suitable substitute for antibiotics, bacteriophage had aroused extensive attention from industry experts and relevant enterprises [8]. Sequencing and analysis of phage genomes were also an important part of phage research[9]. Each gene encoded different protein and thus had a different function. For example, some gene encoded proteins worked as lytic enzymes or lysins, which directly affected the ability of phages to lyse bacteria [10]. The analysis of phage genome was conducive to better understanding the function of phage, and provided a basis for further development and utilization of phage. In our study, we successfully isolated two bacteriophages of *Aeromonas hydrophila*

(PZL-Ah1 and PZL-Ah8) and analyzed their biological characteristics and genomes. We found that these two phages had unique characteristics. Sequencing and analysis with the NCBI database showed that these were two completely new phages. It laid the foundation for the combination of phage cocktail therapy and construction of engineering phage in the future clinical application.

2 Phage Purification And Characteristics

PZL-Ah1 and PZL-Ah8 were isolated from the sewerage systems in Changchun, China. Two distinct plaque morphologies were identified, PZL-Ah1 and PZL-Ah8 phages formed plaques with 2 to 4 mm diameters surrounded by a thin area of secondary lysis of 1 mm. TEM image revealed that virion of PZL-Ah1 and PZL-Ah8 had capsid diameter ≈ 51 ± 1 nm and tail ≈ 21 ± 1 nm (Fig. 1A). The morphological characteristics of PZL-Ah1 and PZL-Ah8 suggested that they both belonged to the *Podoviridae* family (Fig. 1B). The infection of *Aeromonas* bacteria had obvious host specificity, and they could rarely infect extraspecific bacteria. However, there was a certain cross infection rate for intraspecific bacteria. We determined the host ranges of PZL-Ah1 and PZL-Ah8 against a panel of 41 *Aeromonas* spp. strains at a multiplicity of infection (MOI) of 0.1 (PZL-Ah1) and (MOI) of 0.01 (PZL-Ah8) to detect visible plaques. Phage PZL-Ah1 could lyse 7 *Aeromonas* strains, including (4/23) strains of *A.hydrophila* (AH-119, AH-150, AH-87, AH-138), 3/18 strain of *Aeromonas veronii* (AV-6, AV-11, AV-77) (Table 1). For the phage PZL-Ah8, it could also lyse (7/23) *A.hydrophila* strains (AH -119, AH -138, AH -103, AH -68, AH -142, AH -64 and AH -87). By testing, we found that the PZL-Ah1 and PZL-Ah8 could lyse a common *A.hydrophila* strain *A.hydrophila* AH-138.

Table 1. Bactericidal spectrum of PZL-Ah1 and PZL-Ah8

Organism	Bacterial strain	PZL-Ah1	PZL-Ah8	Organism	Bacterial strain	PZL-Ah1	PZL-Ah8
		Efficiency	Efficiency			Efficiency	Efficiency
A.hydrophila	147[C]			*A.veronii*	1[C]		
A.hydrophila	119[C]			*A.veronii*	3[C]		
A.hydrophila	138[C]			*A.veronii*	4[C]		
A.hydrophila	1021[C]			*A.veronii*	5[C]		
A.hydrophila	150[C]			*A.veronii*	6[C]		
A.hydrophila	107[C]			*A.veronii*	7[C]		
A.hydrophila	166[C]			*A.veronii*	9[C]		
A.hydrophila	69[C]			*A.veronii*	11[C]		
A.hydrophila	TPS[C]			*A.veronii*	20[C]		
A.hydrophila	36[C]			*A.veronii*	21[C]		
A.hydrophila	103[C]			*A.veronii*	47[C]		
A.hydrophila	68[C]			*A.veronii*	77[C]		
A.hydrophila	6[C]			*A.veronii*	85[C]		
A.hydrophila	142[C]			*A.veronii*	115[C]		
A.hydrophila	143[C]			*A.veronii*	155[C]		
A.hydrophila	152[C]			*A.veronii*	TH0426[C]		
A.hydrophila	BSK[C]			*A.veronii*	QXF0711B[C]		
A.hydrophila	54[C]			*A.veronii*	520[C]		
A.hydrophila	183[C]						
A.hydrophila	93[C]						
A.hydrophila	87[C]						
A.hydrophila	148[C]						
A.hydrophila	64[C]						

: strains that can be lysed by phages

: strains that cannot be lysed by phages;

C : isolated from the carp kept in our lab (Changchun, Jilin province, China).

3 The Whole Genome Analysis

The complete genome sequences and annotated information of PZL-Ah1 (MT681669.1) and PZL-Ah8 (MZ851152.1) have been submitted to GenBank. The PZL-Ah1 and PZL-Ah8 phage genome were 38,641 bp and 40,855bp contiguous sequence of double-stranded DNA, linear, respectively. The overall G□C content was 53.68% including composition of G (26.11%), C (27.57%), A (22.03%) and T (24.29%) in the gene of phage PZL-Ah1. For the gene of PZL-Ah8, the overall G□C content was 51.89% including composition of G (24.94%), C (26.95%), A (23.23%) and T (24.87%), respectively (Fig. 2A).

BLASTn was used to determine the degree of sequence identity of the two phage genomes and also to other phage genomes in the NCBI nucleotide (nt) database. Through gene comparison in NCBI database showed that PZL-Ah1 and PZL-Ah8 were 97.67% − 95.51% identical to *Stenotrophomonas* phage IME15 and *Aeromonas* Phage T7-Ah. We also used the Emboss Stretcher database to analysed and made the comparison. The result showed that PZL-Ah1 and PZL-Ah8 were 48.2% -48.2% identical to *Stenotrophomonas* phage IME15 and *Aeromonas* Phage T7-Ah. We compared the whole genome of phage PZL-Ah1 with phage PZL-Ah8 and found that although both phages belonged to *Aeromonas hydrophila* phage, only in the range of 16000 − 2400 bp and 32000–40000 bp having relatively similarities (Fig. 2B). The results indicating that the two phages had their own unique sequences.

4 The Function Of Open Reading Frames (Orfs)

Open reading frame (ORF) prediction in assembled contigs was carried out in the GeneMark suite. The functional annotation of proteins encoded by these ORFs were performed using BLASTp, NCBI Conserved Domain Search, HHpred Interactive Server and Inter-ProScan. The phage genomes were also analyzed by tRNAscan-SE to detect tRNA genes. Phage PZL-Ah1 and Phage PZL-Ah8 included with 44 ORFs and 52 ORFs, respectively. The size of proteins encoded by 44 ORFs of PZL-Ah1 ranged from 4.39 kDa (ORF 19) to 143.40 kDa (ORF 06). For the phage PZL-Ah8, the protein sizes ranged from 4.50 kDa (ORF 08) to 150.07 kDa (ORF 01). During the annotation process, 28 (63.6%) ORFs in PZL-Ah1 and 29 (55.8%) ORFs in PZL-Ah8 were homologous to the sequences of known functional proteins in NCBI database. In addition, 16 (36.4 %) ORFs in PZL-Ah1 and 23 (44.2%) ORFs in PZL-Ah8 were classified as “hypothetical proteins” with yet unknown functions (Table S1 and S2).

Like most phages, the coding region of *Aeromonas* phage genome was closely arranged, and the genes related to the function were clustered. In terms of gene functions, except for genes with unknown functions, the genome of *Aeromonas* phage could be divided into three functional categories, so as to the PZL-Ah1 and PZL-Ah8: nucleic acid metabolism and replication module; phage structure and packaging; host lysis. The gene sequence of each module was relatively conservative. PZL-Ah1 and PZL-Ah8 belonged to the *Podoviridae* family. A major difference between *Podoviridae* and *Siphoviridae* was the small number of ORFs, from about 20 to 50. But none of them contain lysogenic modules [11]. The lysogenic module consists of

integrase and repressor proteins that were key to getting the phage into the lysogenic or lytic cycle, no ORFs were associated with drug resistance or lysogeny (e.g., site-specific integrases and repressors) in both genomes (Fig. 3). The nucleic acid metabolism and replication module contains genes related to DNA replication and gene regulation respectively. There were 8 ORFs and 12 ORFs related to nucleic acid metabolism and replication module in PZL-Ah1 and PZL-Ah8. The phage structure and packaging module contains terminal enzymes related to DNA packaging and genes in head and tail morphogenesis. 15 ORFs and 14 ORFs related to the structure and packaging module existed in the PZL-Ah1 and PZL-Ah8 genomes. Endolysin (lysin) proteins, which were found to be encoded in the two phage genomes (ORF 02, ORF 29 and ORF 04) in PZL-Ah1, ORF 24 in PZL-Ah8), were responsible for host cell lysis and release of phage progeny. Due to their ability to kill bacteria by degrading their cell wall, lysins had recently emerged as novel anti-microbial molecules, that could potentially be used to tackle emerging antimicrobial resistance challenges in bacteria [12, 13]. The receptor binding protein (RBP) interaction determined the range and specificity of the phage host. By comparing the ORFs of the two phages, we found the two phages encoded host range proteins (ORF16 in PZL-Ah1, ORF12 in PZL-Ah8). ORF16 in PZL-Ah1 encoded 89 amino acids with a protein size of about 9.15kDa, while ORF12 in PZL-Ah8 encoded 95 amino acids with a protein size of about 10.10 kDa. As for the receptor binding protein, we could use genetic engineering technology to expand the host range. So as to effectively solve the problems of strong specificity and narrow host range of phage application. Bardyt [14] found that when used genetic engineering techniques to modify the host decision region genes of phages, which could broaden the host range of phages. Yoichi [15] exchanged the GP37 and GP38 regions of T2 phage with PP01 phage to form the new recombinant T2 phage, which could infect the heterogeneic *Escherichia coli*, thus effectively expanded the host spectrum of T2 phage.

5 Phylogenetic Tree And Genome Collinearity Analysis

Horizontal gene transfer was thought to play an important role in phage evolution, especially in phage genomic diversity. A total of 34 phages with high homology to these two phages were compared and MEGA7 was used to map the phylogenetic tree (Fig. 4A). As shown in Fig. 4A, the two bacteriophages were on the same large branch with the phage PZL-Ah152 and phage Rostov-1, and the bootstrap in this branch was 100, indicating that the four bacteriophages were closely related. PZL-Ah1, PZL-Ah8 and PZL-Ah152 belonged to the same *Aeromonas* genus, and they were in the same *Vibrio* family as the phage Rostov-1. We performed collinearity analysis of PZL-Ah1 and PZL-Ah8 with two other phages from the same and different branches, respectively (Fig. 4B). As revealed from a comparative analysis, phage PZL-Ah1 and PZL-Ah8 genome were highly similar to that of the *Aeromonas* phage PZL-Ah152 and *Vibrio* phage Rostov-1.

6 Conclusion

Bacteriophages could recognize bacterial cell walls, transfer genetic information, and specifically killed their host strain [16]. In the present study, the genomes of two *A. hydrophila*-specific phages PZL-Ah1 and PZL-Ah8 were isolated, characterized and sequenced. According to gene sequences comparition, their genomes did not exhibit 100% identical sequence homologies to currently known phage strains in GenBank, suggesting that they were two novel phage strains. A total of 114 *Aeromonas* phage genome sequences have been released in GeenBank. The discovery of two novel phages could help us better understand the *A. hydrophila* phage

genome characters, which expanded the diversity of *Aeromonas* phage gene on NCBI. As well as provide reference for application of phage in the future.

Declarations

Accession numbers of nucleotide sequences

The genome sequences of PZL-Ah1 and PZL-Ah8 are available in the NCBI GenBank database under the accession numbers MT681669.1 and MZ851152.1, respectively.

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Compliance with ethical standards

Conflict of interest

All authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

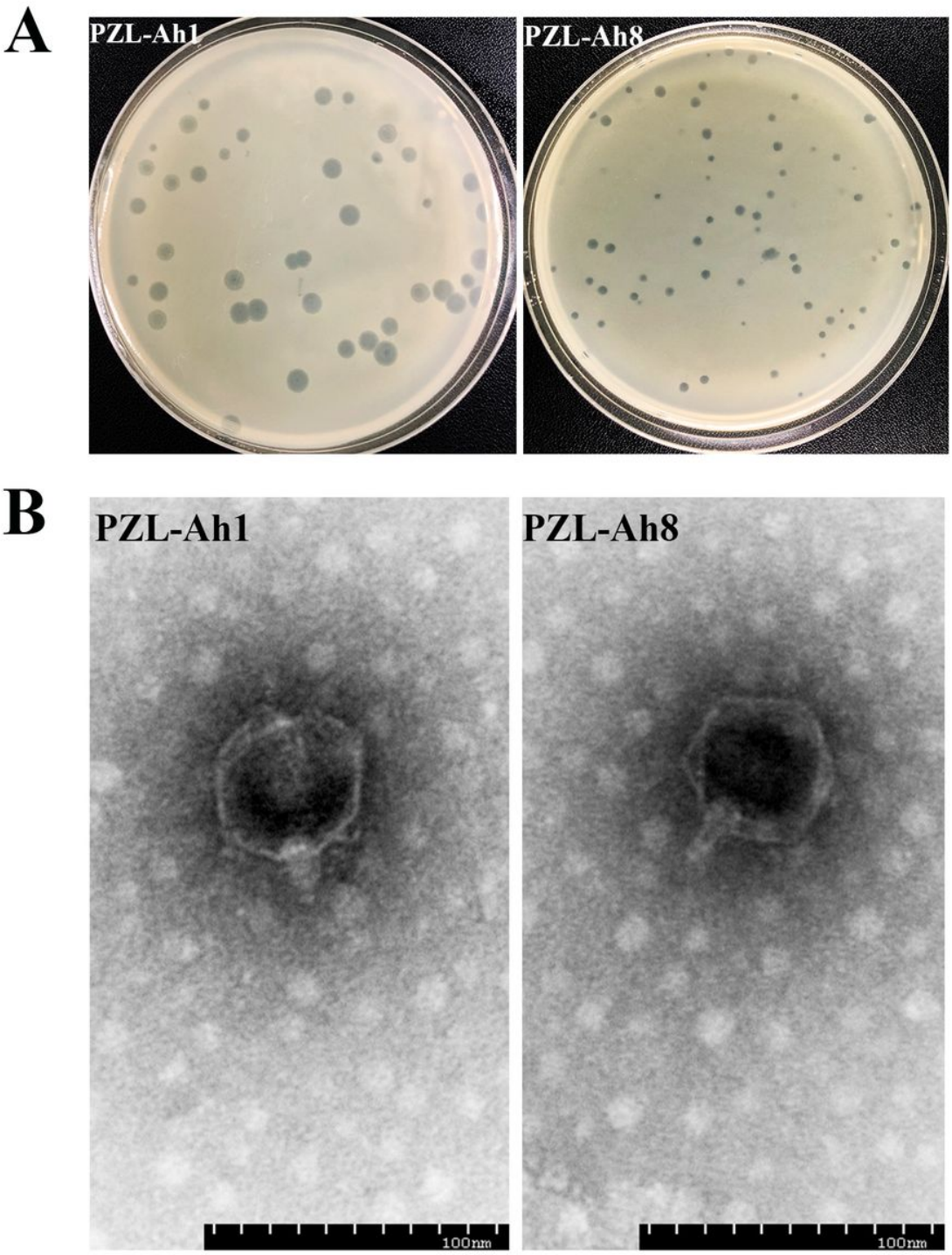

Figure 1

The analysis of two Aeromonas hydrophila morphology. (A) Images of bacterial plaques formed by isolated phage in double agar plate of PZL-Ah1 and PZL-Ah8. (B) Transmission electron micrographs of PZL-Ah1 and PZL-Ah8 stained with 2% phosphotungstic acid.

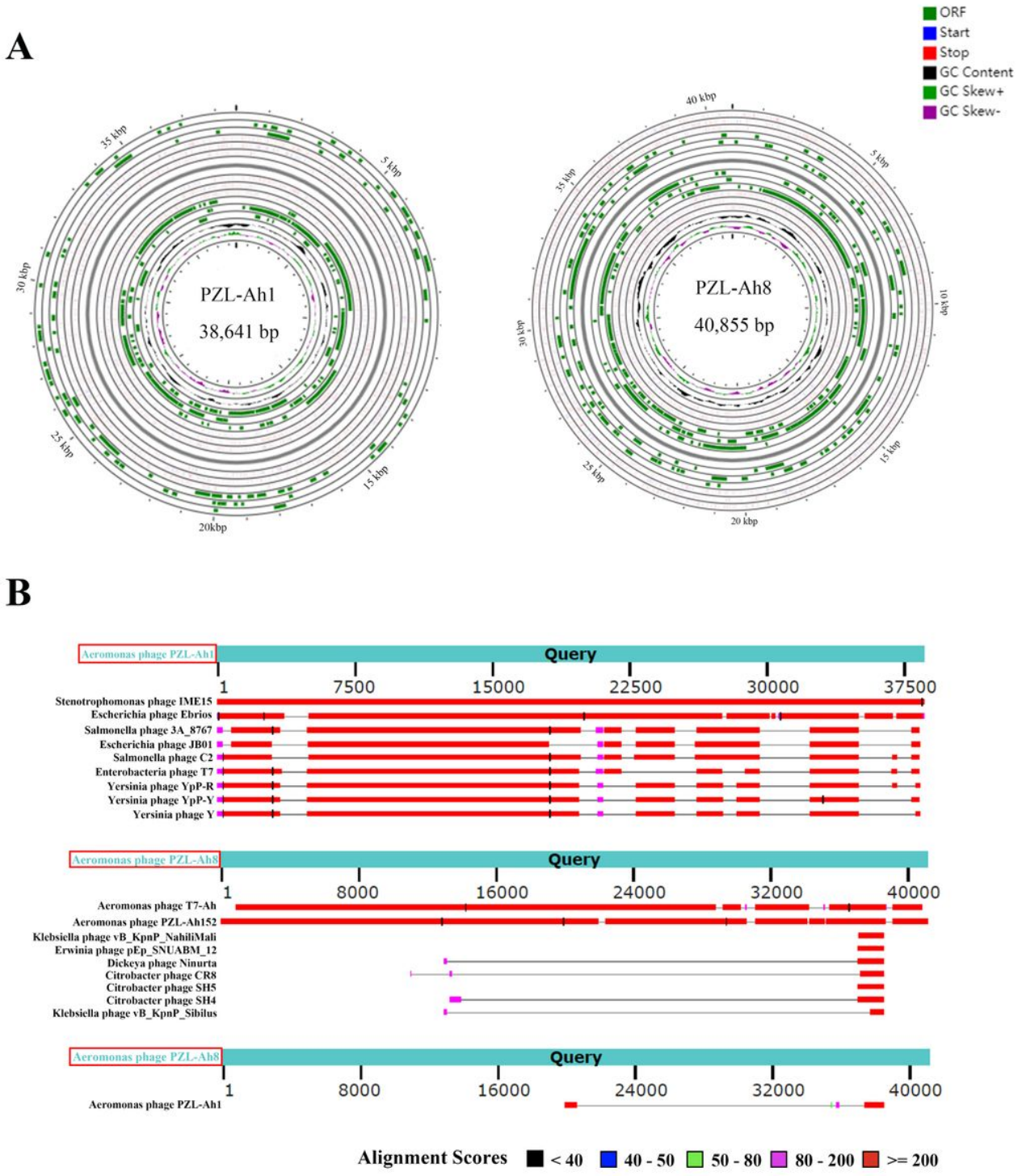

Figure 2

Genome characteristics of phage PZL-Ah1 and PZL-Ah8. (A) The genetic and physical organization of PZL-Ah1 and PZL-Ah8 genome. ORFs with 44 (PZL-Ah1), 52 (PZL-Ah8) or more residues were represented by arrows and the arrowheads pointed in the transcription direction. The figure also mapped G+C content, which was skewed. (B) A general comparison of the genome of PZL-Ah1 and PZL-Ah8 with other phages. All the phage genome sequences involved were obtained from GeenBank.

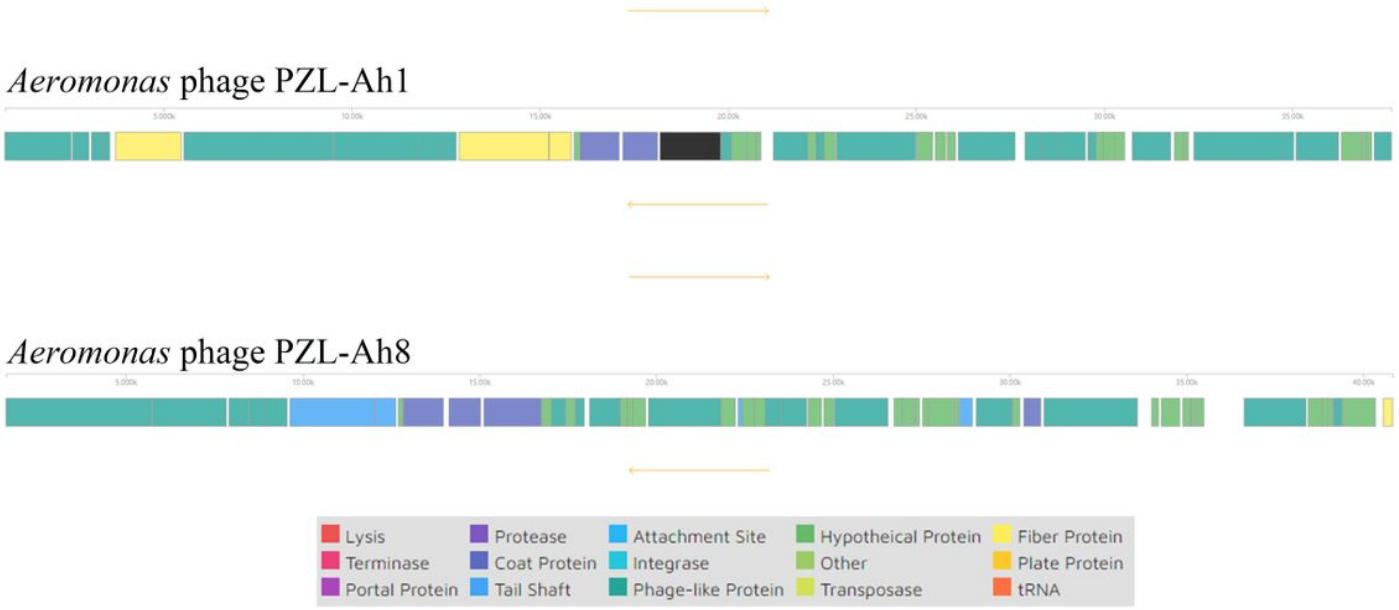

Figure 3

The genetic and physical organization of the PZL-Ah1 and PZL-Ah8 genome. Different colors indicated different function modules, and black indicated unknown function modules.

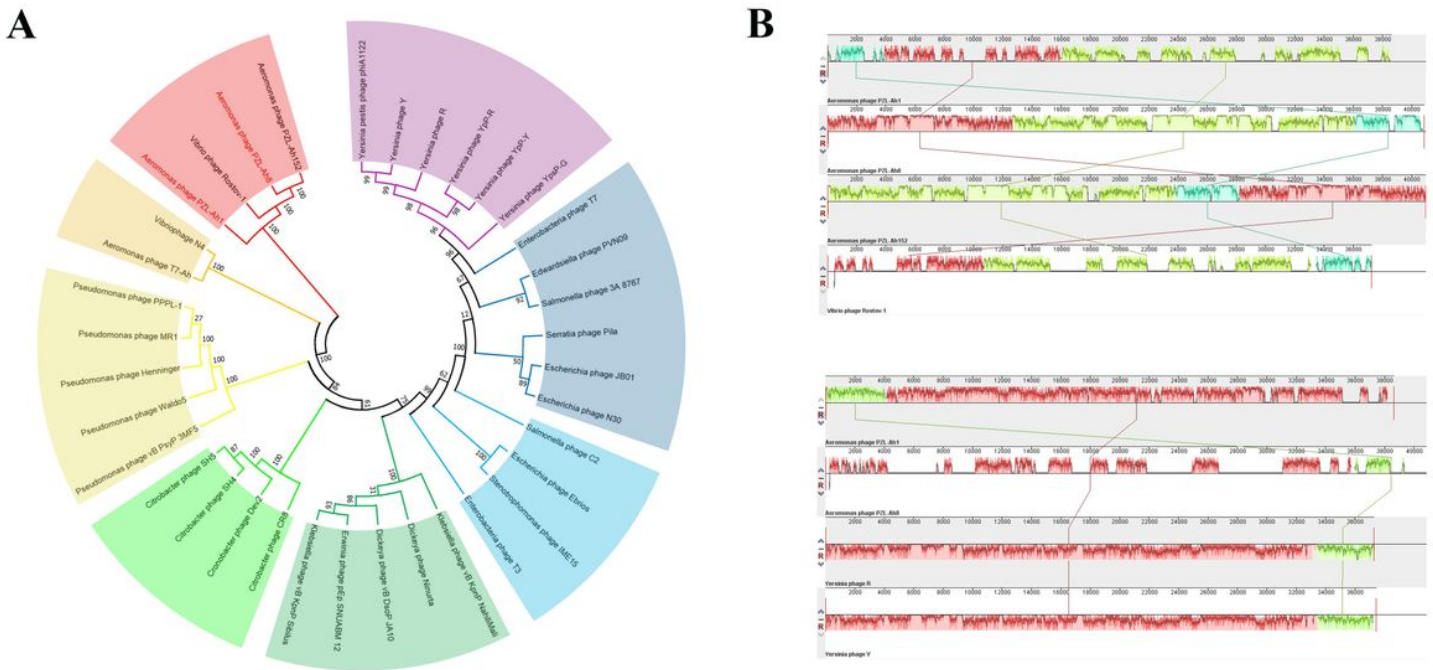

Figure 4

Phylogenetic tree and genome collinearity analysis. (A) Phylogenetic analysis of PZL-Ah1, PZL-Ah8 as well as other phage genome sequences with high genomic similarity available in the NCBI database. (B) Multiple genome alignments among two phages (PZL-Ah1 and PZL-Ah8) and other homologous phages. Similarity

was indicated by the height of the bars, complying with the average level of conservation in that region of the genome sequence. The homologous phages are marked in the picture.

Supplementary Files

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