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Research paper

Isolation, genome sequencing and functional analysis of two T7-like coliphages of avian pathogenic *Escherichia coli*

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ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) causes colibacillosis, which results in significant economic losses to the poultry industry worldwide. Due to the drug residues and increased antibiotic resistance caused by antibiotic use, bacteriophages and other alternative therapeutic agents are expected to control APEC infection in poultry.

Two APEC phages, named P483 and P694, were isolated from the feces from the farmers market in China. We then studied their biological properties, and carried out high-throughput genome sequencing and homology analyses of these phages. Assembly results of high-throughput sequencing showed that the structures of both P483 and P694 genomes consist of linear and double-stranded DNA. Results of the electron microscopy and homology analysis revealed that both P483 and P694 belong to T7-like virus which is a member of the Podoviridae family of the Caudovirales order.

Comparative genomic analysis showed that most of the predicted proteins of these two phages showed strongest sequence similarity to the Enterobacteria phages BA14 and 285P, Erwinia phage FE44, and Kluyvera phage Kvp1; however, some proteins such as gp0.6a, gp1.7 and gp17 showed lower similarity (<85%) with the homologs of other phages in the T7 subgroup. We also found some unique characteristics of P483 and P694, such as the two types of the genes of P694 and no lytic activity of P694 against its host bacteria in liquid medium.

Our results serve to further our understanding of phage evolution of T7-like coliphages and provide the potential application of the phages as therapeutic agents for the treatment of diseases.

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1. Introduction

Infections with avian pathogenic *Escherichia coli* (APEC) cause colibacillosis, an acute and mostly systemic disease that affects the poultry industry worldwide (Ewers et al., 2003). The use of antibiotics as feed additives for growth promotion and disease prevention caused antibiotic resistance (Hasan et al., 2011; Chen et al., 2015; Li et al., 2015), which pose a threat to human health and the environment (Pikkemaat, 2009). The emergence of multidrug resistance among APEC strains has created major economic and health concerns (Mellata, 2013).

The use of bacteriophages for the control of bacterial infections has been considered as an alternative to, or could be used in association with antibiotic therapy (Brussow, 2012; Samson et al., 2013). Bacteriophages are viruses that infect and replicate within bacteria. They have been studied for their potential against multi-drug-resistant strains of many bacteria (Keen, 2012). Phage therapy has many advantages. Their specificity for target bacteria reduces the damage to the normal flora of the host (Clark and March, 2006). Replication at the site of infection is safe with no or few side effects (Sulakvelidze et al., 2001). However, one of the main drawbacks of phages as therapeutic agents is their narrow range in the host (Haq et al., 2012). Therefore, understanding basic features and genomic diversities across phage species could lay the foundation for future applications such as determining the dosage and routes of administration.

In this study, we reported the characteristics and functional genome analysis of two APEC phages; to our knowledge, these phages were the first phages which host was APEC from the T7-like phage family to be reported. Some interesting characteristics of P483 and P694 were reported. Key genes such as tail fiber protein gene were analyzed.

2. Materials and methods

2.1. Bacterial strains and medium

Avian pathogenic *E. coli* strain DE048, DE069 and other 72 *E. coli* strains used in these experiments were isolated from the brains of ducks with clinical signs of sqepticaemic and neurological symptoms

Abbreviations: ORF, open reading frame; DNA, deoxyribonucleic acid; MOI, optimal multiplicity of infection; LB, Luria-Bertani; APEC, avian pathogenic *Escherichia coli*; SDS, sodium dodecyl sulfate; PTA, phosphotungstic acid; TEM, transmission electron microscope; PEG, polyethylene glycol; NCBI, National Center for Biotechnology Information; RNA, ribonucleic acid; LPS, lipopolysaccharides; Ocr, overcomes classical restriction activity.

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Table 1
Escherichia coli strains used in phage isolation and host range analysis.

Strain	Place of isolation	Strain	Place of isolation
DE002	Lishui, Jiangsu	DE186	Jiangning, Jiangsu
DE003	Lishui, Jiangsu	DE192	Quanjiao, Anhui
DE005	Lishui, Jiangsu	DE197	Wuhu, Anhui
DE007	Lishui, Jiangsu	DE205	Bengbu, Anhui
DE010	Lishui, Jiangsu	DE207	Jiangning, Jiangsu
DE011	Lishui, Jiangsu	DE209	Jiangning, Jiangsu
DE015	Lishui, Jiangsu	DE217	Jiangning, Jiangsu
DE017	Lishui, Jiangsu	DE241	Quanjiao, Anhui
DE019	Hexian, Anhui	DE242	Quanjiao, Anhui
DE021	Hexian, Anhui	DE248	Laian, Anhui
DE022	Hexian, Anhui	DE257	Laian, Anhui
DE023	Hexian, Anhui	DE278	Jiangning, Jiangsu
DE031	Hexian, Anhui	DE282	Jiangning, Jiangsu
DE034	Hexian, Anhui	DE283	Jiangning, Jiangsu
DE041	Hexian, Anhui	DE295	Jiangning, Jiangsu
DE048[a]	Quanjiao, Anhui	DE296	Jiangning, Jiangsu
DE049	Quanjiao, Anhui	DE301	Jiangning, Jiangsu
DE069[b]	Liuhe, Jiangsu	DE302	Jiangning, Jiangsu
DE071	Qiligang, Jiangsu	DE303	Jiangning, Jiangsu
DE083	Qiligang, Jiangsu	DE312	Jiangning, Jiangsu
DE104	Liuhe, Jiangsu	DE316	Jiangning, Jiangsu
DE119	Liuhe, Jiangsu	DE322	Jiangning, Jiangsu
DE120	Liuhe, Jiangsu	DE327	Jiangning, Jiangsu
DE123	Liuhe, Jiangsu	DE353	Jiangning, Jiangsu
DE126	Bengbu, Anhui	DE365	Jiangning, Jiangsu
DE127	Bengbu, Anhui	DE373[a]	Jiangning, Jiangsu
DE132	Bengbu, Anhui	DE376	Jiangning, Jiangsu
DE134[a]	Shandong	DE379	Jiangning, Jiangsu
DE137	Shandong	DE384	Jiangning, Jiangsu
DE144	Shandong	DE389	Jiangning, Jiangsu
DE147	Shandong	DE402	Jiangning, Jiangsu
DE160	Guannan, Jiangsu	DE404	Jiangning, Jiangsu
DE167	Jiangning, Jiangsu	DE407	Jiangning, Jiangsu
DE169	Jiangning, Jiangsu	DE426	Chuzhou, Anhui
DE172	Jiangning, Jiangsu	DE452	Chuzhou, Anhui
DE182	Quanjiao, Anhui	DE456	Chuzhou, Anhui
DE183	Quanjiao, Anhui	DE464	Chuzhou, Anhui
K88	Guangzhou, Guangdong	RS218	USA
O138	Yangzhou, Jiangsu	NT01	Nantong, Jiangsu
E1102	Yangzhou, Jiangsu	MC1061[a]	(ATCC 53338)
O157	(ATCC43889)	DH5α[a]	standard strain

[a] *Escherichia coli* strains P483 infect.
[b] *Escherichia coli* strains P694 infect.

at different times and areas in China. All strains were grown in LB liquid medium or LB agar plate at 37 °C.

2.2. Isolation and propagation of bacteriophages

The feces samples were centrifuged twice at 4000 ×g for 10 min at 4 °C, and the supernatant was filtered through 0.22 μm millipore filters and stored at 4 °C. To detect the presence of phage in the filtrate, spot testing was performed (Carlson, 2005). Phages were obtained by the double-layer agar plate method and stored at 4 °C (MH, 1959).

2.3. Electron microscopy

The phage filtrate was applied to a copper grid before negative staining with phosphotungstic acid (PTA, 2% *w*/*v*). Electron micrographs were observed using an H_7650 (Hitachi, Japan) transmission electron microscope (TEM).

2.4. Host range analysis

To investigate the sensitivity of APEC strains to P483 and P694, 80 isolates (Table 1) were used to test the lytic spectrum (Jamalludeen et al., 2007). Cultures of bacteria at log phase (100 μl) were spread over an LB agar plate and allowed to dry. Phage suspensions (10 μl at 10^9 pfu/ml) were dropped in the center of each square and allowed to dry. Following overnight incubation at 37 °C, the plates were examined for lysis.

2.5. Thermo- and pH stability

For thermo stability testing of P483 and P694, samples of the isolated phage were incubated at 45 °C, 50 °C, 55 °C, 60 °C, 65 °C and 70 °C, and were removed at 30 min to be titered by the double-layer agar plate method.

For pH stability testing of P483 and P694, samples of the isolated phage were mixed in a series of tubes containing normal saline, each with a different pH (adjusted using NaOH or HCl), incubated for 1 h at 37 °C, and then tittered by the double-layer agar plate method (Ma and Lu, 2008).

2.6. Optimal multiplicity of infection (MOI)

Multiplicity of infection (MOI) was defined as the ratio of virus particles to potential host cells (Birge, 2000). For MOI testing of P483 and P694, DE048 and DE069 was grown in LB broth at 37 °C to an absorbance at 600 nm of 0.2, measured in a spectrophotometer (Bio-Rad), which corresponded to a cell count of approximately 1×10^8 cfu/ml. The early log phase cells were infected with P483 and P694 respectively at seven different ratios (Table 2). After incubation for 3.5 h at 37 °C, the phage lysate was centrifuged at 6000 ×g for 5 min. The supernatant was diluted to determine the phage titer (Lu et al., 2003). The highest phage growth multiple within 3.5 h was considered as an optimal MOI.

2.7. One-step growth curve

The phage adsorption assay was carried out according to a protocol described previously (Hejnowicz et al., 2009). For one-step growth curve testing of P483 and P694, DE048 and DE069 were grown in LB broth until the optical density at 600 nm reached 0.1, respectively. To 0.9 ml of this culture, 0.1 ml of bacteriophage P483 and P694 (1×10^8 PFU/ml) was added, and incubated at 37 °C for 5 min to allow for adsorption of phages to bacterial cells. The mixture was then centrifuged (6000 *g*, 5 min); the supernatant, containing un-adsorbed bacteriophages, was discarded and the pellet was suspended in 10 ml LB broth. The titer of free unabsorbed phage in the supernatant was then determined by the double-layer agar plate method. Serial dilutions of the pellet were made and incubated at 37 °C. Samples were taken at 2 min intervals, then diluted and plated onto the indicator strain. Plaque forming units were assayed by the double-layer plate method. The burst size was determined as an average yield of phage particles per cell. The latent period was calculated directly from the one-step growth curve obtained. Data represent the results of two independent experiments.

2.8. DNA isolation

Phages were purified with NaCl-polyethylene glycol (PEG) 8000, and DNA was isolated using SDS-Proteinase K (Sambrook and Russell, 2006). To analyze the quality of DNA fragments, the molecules were separated by 0.8% (*w*/*v*) agarose gel electrophoresis in TAE buffer (40 mM Tris–HCl, 500 mM sodium acetate, 50 mM EDTA, pH 7.2).

2.9. Genome sequencing and functional analysis

Genomic sequencing was performed using Ion Torrent and reads were assembled into contigs using the MIRA (version 3.9.9). Sanger sequencing method was used to check the result of Ion Torrent sequencing. Putative open reading frames (ORFs) were predicted and sequence similarity searches and genome analysis were performed by RAST (Aziz et al., 2008; Overbeek et al., 2014; Brettin et al., 2015). The phylogenetic trees of several selected genes were constructed with MEGA5.2 (Tamura et al., 2011) using the Neighbor-Joining algorithm.

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