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LETTER

Characterization and complete genome sequence of vB_EcoP-Bp4, a novel polyvalent N4-like bacteriophage that infects chicken pathogenic *Escherichia coli*

Dear Editor,

Pathogenic *Escherichia coli* cause chicken colibacillosis, which is economically devastating to the poultry industry worldwide (Bagheri et al., 2014). Owing to increasing antibiotic resistance, phage therapy reagents have been developed to treat bacterial infections (Xu et al., 2015).

Coliphage N4 is the first reported phage in the "N4-like viruses" genus and the only member recognized by the International Committee on Taxonomy of Viruses (Schito, 1974). N4-like phages have the characteristic protein DNA-dependent virion RNA polymerase (vRNAP) (Gan et al., 2013). As the largest known protein delivered by a prokaryotic protein transport system, vRNAP is packaged into its capsid and injected together with the first 500 bp of the genomic DNA into the host cell. Thus, N4-like phages transcribe their early genes at the left terminal region of genome using only their own protein machinery (Choi et al., 2008). In this letter, we report the characterization of a novel bacteriophage vB_EcoP-Bp4 (hereafter designated Bp4) isolated from chicken feces in 2005.

Bp4 was propagated in *E. coli* (EC041029A, serotype O78, shown in Supplementary Table S1), and plaque was purified as previously described (Zhang et al., 2013). Plaque assays and spot tests for host range were performed with 24 clinical *E. coli* strains having different serotypes and four laboratory *E. coli* strains; detailed information for these strains is listed in Supplementary Table S1 (Zhang et al., 2013). Bp4 could lyse eight of 24 clinical *E. coli* strains and *E. coli* DH5α, forming clear plaques with an average size of 1–2 mm on double-layered agar (Supplementary Table S1). Bp4 was purified and examined by transmission electron microscopy (JEM-1200EX) (Zhang et al., 2013); the virus had a morphology characteristic of the *Podoviridae* family, with an isometric head of approximately 60 nm in diameter and a tail of approximately 30 nm in length (Figure 1A).

The biological characteristics of Bp4 were tested, as previously described (Zhang et al., 2013). Bp4 could maintain its infectivity after at least 3 h of incubation at pH values ranging from 5.0 to 11.0, but exhibited poor stability outside of this pH range, showing a 100% reduction in infectivity after 1 h of incubation (Figure 1B). Moreover, Bp4 showed stable activity after 1 h of incubation at 40 °C or 50 °C, while the phages were almost inactivated after 20 min of incubation at 80 °C (Figure 1C). The optimal multiplicity of infection (MOI) for phage Bp4 was 0.01, with 4×10^9 PFU/mL production. The latent period was approximately 10–15 min, and the burst size was 32 PFU/cell (Figure 1D), which was very different from other N4-like phages (Schito, 1974; Kulikov et al., 2012; Jun et al., 2014). Phage N4 had a latent period of 180 min with a burst size of 3, 000 PFU/cell, and G7C had a latent period of 40–42 min with a burst size of 500–1 000 PFU/cell; the corresponding data for pSb-1 were 15 min and 152 PFU/cell (Gan et al., 2013).

To further characterize phage Bp4, the phage was purified by polyethylene glycol (PEG)-NaCl and density gradient centrifugation and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 15% gels (Govind et al., 2006; Casas and Rohwer, 2007). Two distinct protein bands were separated and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS; Figure 1E). We confirmed that the 44-kDa protein (band a) was the major coat protein and the 28.4-kDa protein (band b) was the capsid decorating protein. The vRNAP protein was not detected by SDS-PAGE in Bp4.

The genomic DNA of phage Bp4 was sequenced with Ion Torrent PGM and de novo assembled (Invitrogen Trading (Shanghai) Co., Ltd., Shanghai, China). The sequence was deposited in GenBank (Accession No. KJ135004.2). Bp4 was presumed to be linear double-stranded DNA and could be completely digested by DNase I and BAL 31 nuclease but not by RNase A or Mung bean nuclease. The Bp4 genome was 72, 583 bp, with a total G+C content of 42.88%. The whole genome sequence of Bp4 was BLAST searched using the NCBI GenBank database, and the results showed that this phage had 91%–96% homology at the nucleotide level with

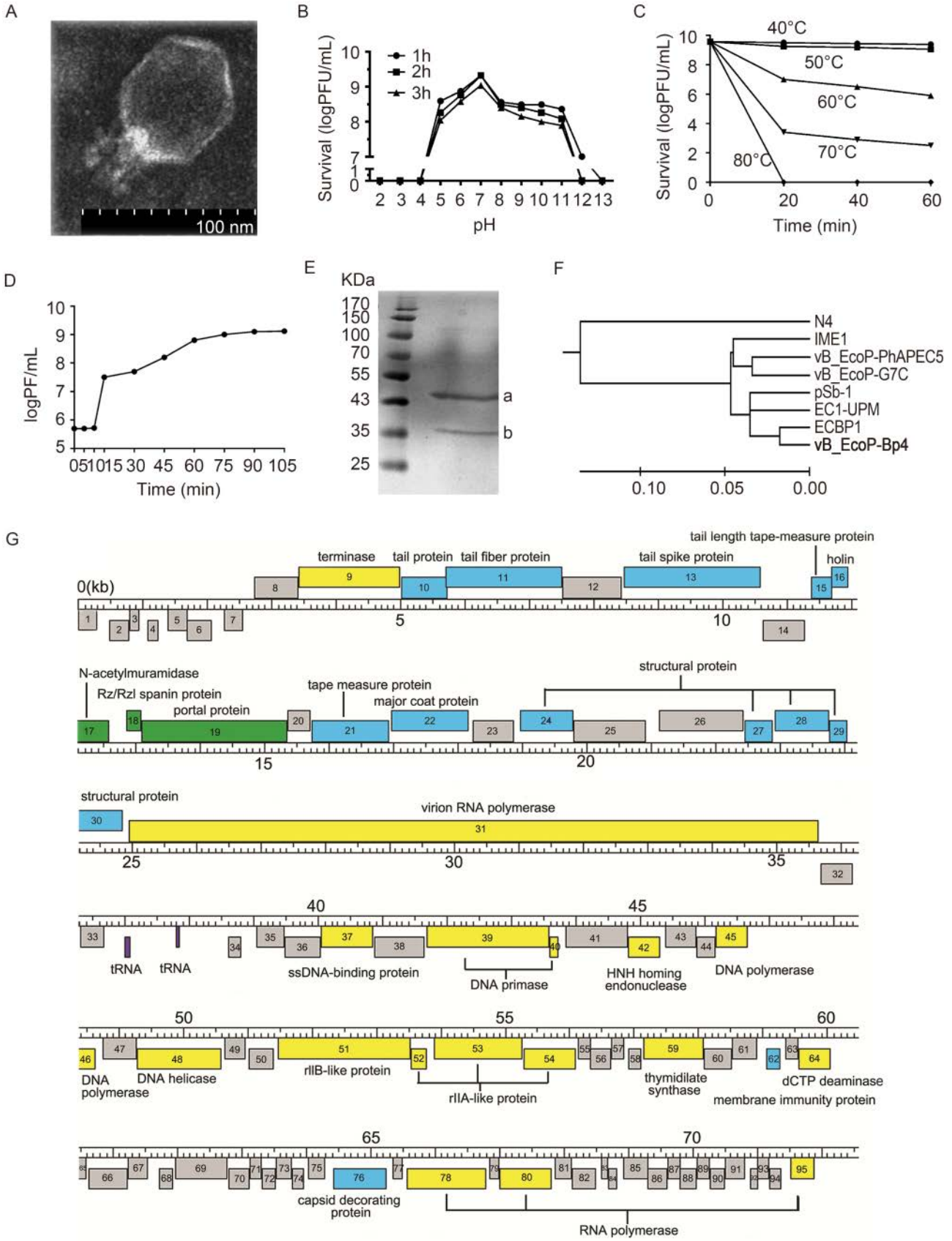
A
100 nm
B
Survival (logPFU/mL)
1h
2h
3h
pH
C
40°C
50°C
60°C
70°C
80°C
Survival (logPFU/mL)
Time (min)
D
logPF/mL
Time (min)
E
KDa
170
150
100
70
55
43
35
25
a
b
F
N4
IME1
vB_EcoP-PhAPEC5
vB_EcoP-G7C
pSb-1
EC1-UPM
ECBP1
vB_EcoP-Bp4
0.10
0.05
0.00
G
0(kb)
terminase
tail protein
tail fiber protein
tail spike protein
tail length tape-measure protein
holin
N-acetylmuramidase
Rz/Rzl spanin protein
portal protein
tape measure protein
major coat protein
structural protein
structural protein
virion RNA polymerase
tRNA
tRNA
ssDNA-binding protein
DNA primase
HNH homing endonuclease
DNA polymerase
DNA polymerase
DNA helicase
rIIB-like protein
rIIA-like protein
thymidilate synthase
dCTP deaminase
membrane immunity protein
capsid decorating protein
RNA polymerase

Figure1. (A) The morphology of phage vB_EcoP-Bp4, as observed by TEM (magnification: 15,000×). (B) The stability of vB_EcoP-Bp4 under different pH conditions. (C) The stability of vB_EcoP-Bp4 under different temperature conditions. (D) One-step growth of phage vB_EcoP-Bp4 in the host strain *E. coli* O78. The latent period was approximately 10–15 min, and the average burst size was approximately 32 PFU/infected cell. The results show the mean values of triplicate experiments. (E) The main proteins of phage vB_EcoP-Bp4 were identified by SDS-PAGE. Lane 1 shows two types of structural proteins labeled with a and b; M: protein molecular weight marker. (F) Evolutionary relationship of phage vB_EcoP-Bp4 with seven other N4-like phages, as analyzed at the genome level. (G) Hypothetical functions of encoded proteins in Bp4 were determined and annotated on the side of the ORFs. Frames with numbers inside indicate the ORFs, and the ORFs on strands + and - were located on the top or bottom of the scale, respectively. Unknown functional ORFs are shown in gray; ORFs associated with structure and packaging are shown in blue; ORFs associated with DNA replication, transcription, modification, and metabolism are shown in yellow; ORFs associated with host lysis are shown in green; and tRNAs are shown in purple. Detailed information on the ORFs is given in Supplementary Table S2.

six other N4-like bacteriophages: ECBP1 (JX415535.1), vB_EcoP-PhAEPC5 (KF192075), IME11 (JX880034), EC1-UPM (KC206276), vB_EcoP-G7C (HQ259105.1), and pSb-1 (KF620435). In contrast, Bp4 displayed 75% homology but only 29% query coverage with phage N4 (EF056009). The genomic terminal sequences of Bp4 were analyzed by next-generation sequencing (NGS) (Li, 2014), and a 411-bp segment (genome positions 72173–72583) was identified as the terminal repeats. Potential open reading frames (ORFs) were predicted and annotated using DNAMaster (http://cobamide2.bio.pitt.edu/) with default parameters. In total, 95 ORFs were predicted, including 35 functional protein ORFs and another 60 ORFs with unknown function (Supplementary Table S2). The genomic map is shown in Figure 1G.

The Bp4 genome was analyzed in a pairwise manner with seven bacteriophages using MEGA to construct the phylogenetic tree (neighbor joining method with 1000 bootstrap repetitions). The results showed that N4 belonged to an exclusive clade, and that Bp4 and the other six phages mentioned above belonged to the same clade (Figure 1F). These findings are consistent with a previous report showing that Bp4 and the other six bacteriophages should belong to a new genus of *G7c virus* and that N4 is actually a genomic orphan (Wittmann et al., 2015).

Two tRNA genes ($tRNA^{Ile}$ and $tRNA^{Cys}$) were found in Bp4 by tRNAscan-SE v1.23 (Lowe and Eddy, 1997); four tRNAs existed in N4, and one was found in pSb-1, whereas no tRNAs were found in IME11, G7C, ECBP1, phAPEC5, and EC1-UPM (Gan et al., 2013). Bailly-Bechet *et al*. (2007) suggested that the number of tRNAs may be responsible for the latent period in N4-like phages. However, there were no obvious relationships between the number of tRNAs and the latent period among N4-like phages in our study.

The genome of Bp4 contained the N4-like phage gene *vRNAP*. Three ORFs in the Bp4 genome, i.e., *orf16* (holin protein), *orf17* (N-acetylmuramidase), and *orf18* (Rz/Rz1 spanin protein), were found to be responsible for host lysis. There was no holin gene homolog in ECBP1 or phAPEC5, no N-acetylmuramidase gene homolog in EC1-UPM, and no spanin gene homolog in pSb-1. These differences indicated that phage lytic activity varied among N4-like phages. Three ORFs in the Bp4 genome, i.e., *orf10* (putative tail protein), *orf11* (tail fiber protein), and *orf13* (tail spike protein), which is responsible for host receptor recognition and adsorption, were identified as tail fiber-associated genes. Notably, *orf11* and *orf13* were deleted in N4. In contrast, *gp65* (tail sheath protein) in N4, which is essential for recognition of the host receptor, was deleted in Bp4 (Supplementary Table S2). These results showing different tail fiber-associated genes in Bp4 and N4 indicated that these phages had different mechanisms of adsorption. Moreover, the Bp4 genome lacked an N4 *gp8* homolog. In the N4 genome, gp8 contributes to phage yield but is not essential for phage growth (Yano and Rothman-Denes, 2011).

Based on our analysis, Bp4 and other N4-like phages should proliferate through different mechanisms. Our findings provide insights into the characteristics of N4-like phages and phage therapy for controlling drug-resistant *E. coli* in chickens.

FOOTNOTES

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Supplementary tables are available on the websites of *Virologica Sinica*: www.virosin.org; link.springer.com/journal/12250.

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