Characterization and Genome Sequencing of a Novel Coliphage Isolated from Engineered *Escherichia coli*

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Key Words

Bacteriophages • Coliphage • Engineered *E. coli* phage •
Genome sequencing • Host range

Abstract

Objectives: To characterize morphological, physicochemical and genomic features of a novel virulent coliphage which was isolated from an engineered *Escherichia coli* culture and termed engineered *E. coli* phage (EEP). **Methods and Results:** Electron microscopy revealed that EEP has an icosahedral head (62 nm in diameter) and a long, flexible tail (138 nm in length). EEP was able to infect all 10 engineered *E. coli* strains kept in our laboratory, showing a strong ability to lyse engineered *E. coli*. Sequencing of the EEP genome revealed a double-stranded DNA (39.8 kb) with 54.72% GC content. Fifty-two open reading frames were predicted to be coding sequences, 18 of which were functionally defined and organized in a modular format, which includes modules for DNA replication, DNA packaging, structural proteins and host cell lysis. This phage could not be inactivated at 90° for 45 min and was resistant to ethanol and alkali treatment. EEP is assigned to the Siphoviridae family based on its morphological, genomic and physicochemical properties. **Conclusions:** A novel coliphage was isolated from engineered *E. coli*

strains, and its morphological, genomic and physicochemical properties were characterized, which will improve our knowledge of bacteriophage diversity.

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Introduction

Bacteriophages are the most abundant biological entities on the planet [1] and are not only highly diverse among themselves, but also result in the diversity of other organisms through the horizontal transference of genetic material, such as bacterial virulence genes [2–5]. Bacteriophages have therefore become an excellent model system and an effective biological tool for understanding fundamental biological phenomena. Although there are approximately 10^{31} different bacteriophages in the biosphere [6], only about 5,500 have been examined using electron microscopy [7], and only 554 phage genomes have been sequenced to date, even fewer than those of bacteria. Isolation and identification of a novel bacteriophage will therefore provide new data on bacteriophage biodiversity, while sequencing of the bacteriophage genome will improve our understanding of phage and bacterial evolution.

Genetically engineered bacteria are also important tools and materials in the field of molecular biology, and have been extensively used in research and bioprocessing industries. Bacteriophage infection of engineered bacteria could have serious consequences due to its inhibition of the fermentation process. Consequently, the fermentation process may be slowed or completely stopped, thereby reducing the quality of the final products. Among the engineered bacteria, *Escherichia coli* is the most frequently used. However, there have been few studies on phages of engineered *E. coli*, except for that of Iida et al. [8], and no engineered *E. coli* phage genome sequence has yet been published, although about 70 Enterobacteria phages have been completely sequenced.

The phage EEP reported here was occasionally found on agar plates of engineered *E. coli* BL21 (DE3). This phage was able to lyse all 10 tested engineered *E. coli* strains and demonstrated a potent lytic ability for engineered *E. coli*. Investigation of the biological traits and whole genome sequence of EEP will lead to a better understanding of this new bacteriophage and devise effective control procedures to alleviate the devastating consequences of its attack in bioprocessing industries and in laboratory environments.

Materials and Methods

Strains and Phages

EEP was occasionally isolated during an electroporation experiment in which engineered *E. coli* strain BL21 (DE3) was used as competent cell. BL21 (DE3) cells were subsequently used as host cells for EEP. Ten genetically engineered *E. coli* strains, including BL21 (DE3), DH10B, DH5 α , JM109, M15, Rosetta, Rosetta-gami, S17-1, S17-1 λ pir⁺ and TOP10, and *E. coli* ATCC 35218 were purchased. The susceptibilities of all the strains to EEP were investigated.

Propagation and Purification of Phages

EEP particles were added to BL21 (DE3) cultures at 0.01 multiplicity of infection and incubated for a further 12–16 h after infection. Crude phage suspensions were prepared by centrifugation of the cultures (10,000 g for 15 min at 4°) to remove the cells and by passing the supernatants through a membrane filter (0.45- μ m pore size). This method yielded phage titers as high as 10¹⁰–10¹¹ plaque-forming units (PFU)/ml. Crude phage suspensions were concentrated with 10% polyethylene glycol 8000 and 1 M NaCl, according to the method of Govind et al. [9]. The precipitate was further purified using CsCl gradient ultracentrifugation [10, 11].

Electron Microscopy

CsCl-purified EEP suspensions (20 μ l), at a concentration of 10¹²–10¹³ PFU/ml, were deposited onto carbon-coated copper grids and allowed to adsorb for 15 min. The phage particles were

negatively stained using 2% (w/v) potassium phosphotungstate (pH 7.2) and examined using a Philips TECNAI 10 electron microscope (Philips, The Netherlands).

One-Step Growth Curve

For one-step growth experiments, a modification of the method of Lu et al. [12] was used, with 15-min adsorption at 37°. Following centrifugation at 13,000 g for 30 s, the pelleted cells were resuspended in 5 ml preheated Luria-Bertani broth, followed by incubation at 37°. Samples were taken at 10-min intervals (up to 2 h) and immediately titrated by PFU counting assay [13]. Experiments were repeated three times with duplicate samples.

Physicochemical Stability of EEP

Resistance of EEP to different temperatures, ethanol levels and pH values was determined, according to the methods of Capra et al. [14]. All results were expressed as the concentration (PFU/ml) of active viral particles and its log₁₀ plotted against time.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and N-Terminal Sequencing of Phage Proteins

CsCl-purified EEP samples were mixed with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer and heated in a boiling water bath for 3 min, then electrophoresed in a 12% SDS polyacrylamide gel. The gel containing the separated protein bands was transferred onto a polyvinylidene fluoride membrane (Millipore, Germany) for sequencing of N-terminal amino acids using an Applied Biosystems Procise 491 protein sequencer (Applied Biosystems, USA; Service Center of Protein Sequencing, Beijing University).

Preparation of Phage DNA

EDTA was added to the purified phage stock solution at a final concentration of 20 mM, proteinase K at 50 mg/ml, and SDS at 0.5% (w/v). The mixture was incubated at 56° for 1 h, and then purified by phenol extraction and ethanol precipitation. Finally, EEP genomic DNA was stored at 4° for use.

Genomic Sequencing and Analysis

Purified EEP genomic DNA was sequenced at the Beijing Genomics Institute, using methods described previously [15–17]. Briefly, phage DNA was mechanically sheared and size fractionated by agarose gel electrophoresis. Fragments 1.6–2.0 kb in size were recovered. The fragments were then digested with mung bean nuclease and ligated to phosphatase-treated vector pUC18/*Sma*I/BAP. The ligated plasmids were electronically transferred to competent *E. coli* DH5 α . The transformed cells were extracted using a Prep 96 plasmid kit and used as DNA sequencing templates. The sequencing work was completed using an Abi PrismTM 377 auto DNA sequencer (Perkin Elmer, USA).

Poor-quality data and vector sequences were removed from the automated sequencing results and the remaining sequences were assembled into contigs using the Phred/Phrap/Consed software package (Genomic Center, Washington University, USA). Open reading frames (ORFs) were analyzed using ORF Finder at the NCBI (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) and putative genes were analyzed using GeneMark.HMM (<http://opal.biology.gatech.edu/GeneMark/genemark24.cgi>). Nucleotide sequences and protein sequences were scanned for homologues using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Fig. 1. Electron micrograph of EEP. Purified phage particles were stained with potassium phosphotungstate and observed in a transmission electron microscope. Bar = 500 nm. The phage particle has an icosahedral head (62 nm in diameter) and a long, flexible tail (138 nm in length).

Fig. 2. One-step growth curve of EEP on BL21 (DE3). Experiments were repeated three times with duplicate samples.

Table 1. Susceptibility of different bacterial strains to EEP

Bacterial strains		Source	Susceptibility
Engineered <i>E. coli</i> strains	BL21 (DE3)	derivative of <i>E. coli</i> B strain with DE3	+
	DH10B	derivative of <i>E. coli</i> MC1061 strain, an <i>E. coli</i> K-12 strain	+
	DH5 α	derivative of <i>E. coli</i> Hoffman-Berling 1100 strain, an <i>E. coli</i> K-12 strain	+
	JM109	derivative of <i>E. coli</i> Hoffman-Berling 1100 strain, an <i>E. coli</i> K-12 strain	+
	M15 (PREP4)	derivative of <i>E. coli</i> K-12 strain	+
	Rosetta (DE3)	derivative of <i>E. coli</i> B strain with DE3	+
	Rosetta-gami (DE3)	derivative of <i>E. coli</i> K-12 strain with DE3	+
	S17-1	derivative of <i>E. coli</i> K-12 strain	+
	S17-1 λ pir ⁺	derivative of <i>E. coli</i> K-12 strain	+
	TOP10	derivative of <i>E. coli</i> MC1061 strain, an <i>E. coli</i> K-12 strain	+
ATCC <i>E. coli</i> strain	ATCC 35218	commercial strain	–

+ = Sensitive; – = insensitive. All bacteria were kept in our laboratory.

Nucleotide Sequence Accession Number

The genomic sequence of EEP has been deposited in GenBank with the accession No. FJ750948 (Enterobacteria phage SSL-2009a, NC_012223).

Results

Morphological Features of EEP

When EEP was grown on agar plates with BL21 (DE3), round, clear phage plaques 1–3 mm in diameter were seen, showing the characteristics of virulent bacteriophages. Electron microscopy showed that EEP virions

have icosahedral heads 62 ± 5 nm in diameter and long, flexible tails 138 ± 16 nm in length (fig. 1).

Physiological Features of EEP

One-Step Growth Curve. The one-step growth curve of EEP was measured on engineered *E. coli* strain BL21 (DE3). As shown in figure 2, latent and burst periods of 10–15 and 30–40 min, respectively, were observed, indicating that only a short time was needed for EEP to adapt to a new environment. The burst size was estimated at 375 ± 43 PFU per infected cell.

Fig. 3. Tolerance of EEP to thermal treatment (mean of 3 determinations).

Fig. 4. Tolerance of EEP to ethanol (mean of 3 determinations).

Host Range. As shown in table 1, EEP was able to infect all the tested engineered *E. coli* strains, revealing a potent lytic capability for engineered *E. coli*, but was unable to lyse the ATCC 35218 *E. coli* strain.

Phage Stability. EEP is relatively resistant to thermal treatment (fig. 3). Heating to 63, 72, 80 or 90° was unable to completely inactivate high-titer phage suspensions, even after 45 min. At 95°, the phage titers were reduced by 6 log₁₀ after only 5 min. Ethanol is a widely used disinfectant. When phage EEP suspensions were treated with ethanol (fig. 4), phage titers were slightly decreased in the presence of ethanol (25, 50 and 75%, v/v), and >100 viable phages per milliliter were detected after treatment with absolute alcohol (100%, v/v) for 45 min. The effect of pH on EEP viability is summarized in figure 5. EEP was able to maintain its infectivity when incubated at 37° in a pH range between 5 and 11, but no phages could be detected after incubation at pH 2–3 for 30 min or at pH 4 for 60 min (<10 PFU/ml).

Genomic Features of EEP

General Features of the EEP Genome. Whole-genome sequencing revealed that the double-stranded genomic DNA (dsDNA) of EEP consisted of 39,792 bp with a nucleotide composition of A (21.34%), G (26.56%), T (23.93%) and C (28.16%). It had an average GC content of 54.72%, which is slightly higher than that of *E. coli* (50.8%) [18]. A total of 223 ORFs >100 bp were predict-

Fig. 5. Tolerance of EEP to pH variation (mean of 3 determinations).

ed from the EEP genome sequence using the software ORF Finder, among which 52 ORFs were identified as coding sequences (CDS). The average length of a gene was about 707 bp. We estimated that 92.34% of the whole sequence was involved in encoding proteins. Bacteriophages use their genetic sequence more efficiently and

Table 2. The genes of EEP with predicated function

Coding ORFs	Strand		Length aa	Mass kDa	Predicated function	E value
1	–	149–844	231	26.46	phage-related hypothetical protein of Enterobacteria phage phi1 p189	1e–77
2	–	841–2,265	474	53.47	phage-encoded associated helicase of <i>Salmonella</i> phage SETP3	2e–170
3	–	2,270–2,494	74	8.68	unknown gene	
4	–	2,532–2,705	57	6.70	unknown gene	
5	–	2,746–3,024	92	10.27	phage-related hypothetical protein of <i>Salmonella</i> phage KS7	2e–17
6	–	3,021–3,212	63	6.92	phage-related hypothetical protein of phage BP-4795	3e–16
7	–	3,217–5,508	763	86.26	phage-related DNA polymerase I of <i>Salmonella</i> phage SETP3	2e–174
8	–	5,505–5,675	56	6.21	unknown gene	
9	–	5,735–6,529	264	30.55	phage-related hypothetical protein of <i>Salmonella</i> phage SETP3	3e–28
10	–	6,605–6,847	80	9.23	unknown gene	
11	–	6,840–8,270	476	52.42	phage-related hypothetical protein of <i>Salmonella</i> phage SETP3	3e–111
12	–	8,270–8,458	62	7.21	unknown gene	
13	–	8,459–8,710	83	9.95	unknown gene	
14	–	8,759–9,301	180	19.77	unknown gene	
15	+	9,702–10,031	109	12.42	unknown gene	
16	+	10,042–12,306	754	82.81	putative phage replicative helicase/primase of <i>Burkholderia pseudomallei</i> BCC215	4e–10
17	+	12,604–12,723	39	4.34	unknown gene	
18	+	12,777–12,995	72	7.92	phage superinfection exclusion protein (COR-like protein) of Enterobacteria phage N15	2e–08
19	–	13,012–13,683	223	23.20	phage-related hypothetical protein of Enterobacteria phage N15	5e–54
20	–	13,686–13,988	100	10.17	phage-related hypothetical protein of Enterobacteria phage HK022	9e–38
21	–	14,026–17,445	1,139	125.74	putative phage tail fiber protein of Enterobacteria phage HK97, N15, T1, etc.	0.0
22	–	17,442–18,059	205	21.31	putative phage tail assembly protein I of <i>Yersinia pestis</i> CO92, KIM, etc.	3e–37
23	–	18,050–18,790	246	27.58	putative phage minor tail protein of <i>Yersinia enterocolitica</i> subsp. enterocolitica 8081	4e–44
24	–	18,793–19,581	262	28.78	phage minor tail protein L of <i>Y. pestis</i> KIM, Angola, etc.	2e–46
25	–	19,578–20,177	199	21.51	putative minor tail protein of Enterobacteria phage T1	1e–06
26	–	20,214–22,853	879	93.15	putative bacteriophage tail tape measure protein of <i>Shigella flexneri</i> 2a str. 301	2e–54
27	–	23,238–23,333	31	3.62	unknown gene	
28	–	23,552–23,956	134	15.53	phage-related hypothetical protein of <i>Y. enterocolitica</i> subsp. enterocolitica 8081	4e–13
29	–	23,985–24,710	241	25.65	putative major tail protein of Enterobacteria phage T1	2e–26
30	–	24,772–25,194	140	15.15	unknown gene	
31	–	25,194–25,787	197	21.95	phage-related hypothetical protein of <i>Proteus mirabilis</i> HI4320	2e–28
32	–	25,789–26,148	119	13.14	conserved phage structural protein of <i>P. mirabilis</i> HI4320	8e–11
33	–	26,247–26,777	176	18.93	unknown gene	
34	–	26,806–27,789	327	34.33	conserved phage structural protein of <i>Pseudomonas</i> phage 73	2e–17
35	–	27,791–27,904	37	4.13	unknown gene	
36	–	28,002–28,703	233	25.35	phage-related hypothetical protein of <i>Pseudomonas</i> phage 73	3e–19
37	+	28,843–29,172	109	11.59	unknown gene	
38	+	29,212–29,790	192	21.87	unknown gene	
39	+	29,804–29,968	54	6.03	lysozyme	4e–18

Table 2 (continued)

Coding ORFs	Strand		Length aa	Mass kDa	Predicated function	E value
40	+	29,985–30,386	133	13.52	phage-related hypothetical protein of <i>Y. pestis</i> CO92	4e–07
41	+	30,621–30,734	37	4.31	unknown gene	
42	+	30,799–31,941	380	42.68	phage-related hypothetical protein of <i>Salmonella</i> phage SETP3	7e–104
43	+	32,051–32,311	86	9.90	unknown gene	
44	+	32,217–32,624	135	14.73	phage-related hypothetical protein of <i>Salmonella</i> phage SETP3	7e–11
45	+	32,624–34,009	461	52.21	phage terminase large subunit of <i>Salmonella</i> phage SETP7	7e–81
46	+	34,021–35,541	506	55.14	conserved phage structural protein of <i>Pseudomonas</i> phage 73	9e–52
47	+	35,525–36,628	367	41.10	phage head morphogenesis protein of <i>Silicibacter</i> sp. TM1040	7e–32
48	+	36,621–36,950	109	11.95	phage-related hypothetical protein of <i>Salmonella</i> phage KS7	2e–07
49	+	36,979–37,062	27	3.03	unknown gene	
50	–	37,076–37,840	254	28.95	phage-related hypothetical protein of <i>Pseudomonas</i> phage YuA	2e–06
51	+	37,893–38,123	76	8.69	unknown gene	
52	–	38,150–39,667	505	55.29	putative tail tip protein of Enterobacteria phage ES18	4e–14

Table 3. The N-terminal amino acid sequencing results of some structural EEP proteins

Protein bands	Sequence of the N-terminal 5 amino acids
123.5 kDa	Met-Ile-Thr-Thr-Tyr (MITTY)
90.2 kDa	Ala-Asp-Gln-Ala-Ala (ADQAA)
65.5 kDa	Thr-Val-Asn-Val-Asp (TVNVD)
40.5 kDa	Ser-Leu-Thr-Val-Phe (SLTVF)
26.3 kDa	Gly-Tyr-Gln-Leu-Pro (GYQLP)
15.6 kDa	Met-Leu-Asp-Ser-Ile (MLDSI)

economically than host bacteria and higher organisms [17, 18].

Predicted CDS and Their Features. When BLASTN analysis of the entire EEP genomic sequence was conducted, no homologous sequences >100 bp were found in the public databases, indicating that the EEP genome is distinct from all previously sequenced phage genomes. The 52 potential CDS of the EEP genome were searched individually using BLASTP at the NCBI database. Positions, sizes and putative functions of the proteins as well as similarities to other proteins are listed in table 2 and figure 6. Among these potential CDS, 18 ORFs (34.6%) identified homologous genes coding for phage structural proteins or replication-related enzymes. The remaining ORFs are functionally unknown, 19 ORFs (36.5%) code for proteins with a high degree of homology to phage-

related hypothetical proteins, and 15 ORFs (28.8%) code for unique products with little or no homology to proteins from the database.

Structural Proteins of EEP and Their N-Terminal Sequencing. In order to analyze the structural proteins of EEP, purified phage particles were treated by boiling with sample buffer, and then separated by SDS-PAGE. At least eight distinct protein bands, with molecular weights ranging from 16 to 124 kDa, were visualized in the gels (fig. 7). The most abundant was a 40.5-kDa protein, most likely to be the major coat protein of EEP, while a 26.3-kDa protein was identified as the secondary coat protein. To determine the coding ORF of every structural protein, all the protein bands in the SDS-PAGE gel were transferred onto polyvinylidene fluoride membranes and their five N-terminal amino acids were sequenced. The results of six bands (123.5, 90.2, 65.5, 40.5, 26.3 and 15.6 kDa) with large enough protein samples are listed in table 3.

Discussion

Bacteriophages are ubiquitous in nature. According to morphology and nucleic acid properties, phages may be divided into 21 morphotypes which correspond to 13 phage families. About 96% of phages investigated in the last 50 years are tailed phages belonging to the Siphoviridae, Myoviridae or Podoviridae families [19, 20]. Siphoviridae (isometric head and non-contractile tail) is by far

Fig. 6. The organization of predicted genes of EEP.

the most frequent phage group (61.7%) [21, 22]. EEP, which has an icosahedral head and a long, flexible and non-contractile tail, should be classified as a member of the Siphoviridae family.

It is known that bacteriophages infect host cells with very high specificity. Phage EEP was isolated from an engineered *E. coli* culture, and we therefore determined which engineered *E. coli* strains were sensitive to EEP. Ten engineered *E. coli* strains that are commonly used in bioprocessing and molecular biological researches were tested for their susceptibilities to phage EEP. Results indicated that all the tested engineered *E. coli* strains were sensitive to EEP, suggesting that EEP has strong lytic ability for engineered *E. coli* strains and could potentially pose a significant threat to biomanufacturing processes that use engineered *E. coli* as the production strain. The most likely explanation for the broad host range of EEP is that some common proteins on the cell surfaces of these host strains are used by EEP as recognition receptor. Among these sensitive engineered *E. coli* strains, BL21 (DE3) and Rosetta (DE3) are derived from *E. coli* B strain, while DH10B, DH5 α , JM109, M15, Rosetta-gami, TOP10, S17-1 and S17-1 λ pir⁺ are from *E. coli* K-12 strain. All these host strains belong to *E. coli* species of the *Escherichia* genus. Therefore, it is not hard to understand that they share common adsorption receptors to phage EEP. Actually, relatively broad host ranges of bacteriophages are also found in other works [13, 23–25].

Fig. 7. EEP proteins separated by SDS-PAGE and their corresponding putative ORFs. Purified EEP particles were lysed in sample buffer and the proteins resolved on a 12% SDS-PAGE gel, followed by Coomassie staining, which revealed at least 8 bands.

Different physicochemical parameters, such as heat, biocide and pH changes of the environment, can affect the viability of phages. Our studies indicated that EEP was able to survive high thermal treatments. The results of thermal tolerance of EEP were similar to those of the *Lactobacillus paracasei* phage (MLC-A), which also demonstrated high resistance to temperatures $>72^{\circ}$ and was completely inactivated when heated at 90° for 2 min [26]. Most bacteriophages are sensitive to thermal treatment: about 76% of phage V-45, a bacteriophage of *Vibrio fetus*, were inactivated by incubation at 20° for 1 h. Proportionately greater inactivation was observed from 30 to 50° , and all phage activity was destroyed after 10 min at 60° [27]. Bacteriophages resistant to high temperatures pose a greater threat to industrial fermentation processes because they are able to survive thermal treatments and multiply at relatively high temperatures.

Ethanol is widely used in disinfection, but the most effective concentration of ethanol (75%, v/v) and even dehydrated alcohol were not able to completely inactivate EEP. The failure of ethanol also suggests the absence of a lipid envelope outside the capsid of the phage.

The data of pH resistance test suggest that EEP is sensitive to acid conditions but tolerant to alkaline conditions. Musarrat and Ahmad [28] reported that bacteriophage λ -*E. coli* complexes exhibited remarkable sensitivity to alkaline pH 10.0 at 37° . Studies of five lactic *Streptococcus* phage strains showed that the proliferation of phage F55 was reduced at pH 7.5, while F24 caused mass lysis of susceptible cells at the same pH level, and strains F56 and F43 were able to proliferate at pH 8.4 and 8.6, respectively [29]. Compared to these bacteriophages, EEP is more tolerant to alkaline conditions.

We determined the nucleotide sequence of the entire EEP genome. Putative ORFs ($n = 52$) were identified as CDS on the EEP genome, and 18 ORFs code for proteins with a high degree of homology to known phage proteins. Generally, phage genomes are organized in modular structures, with each module containing clusters of genes with specific functions [30]. The 18 ORFs of the EEP genome with predicted function are organized into three modules containing gene clusters for DNA replication and DNA packaging, structural proteins and host cell lysis.

ORFs coding for putative phage replicative helicase/primase (ORF2/ORF16), phage-related DNA polymerase I (ORF7) and phage terminase (ORF45) are identified as members of the DNA replication and packaging module of the EEP genome. Primase/helicase is known to be an enzyme with dual activities. During DNA replication,

duplex DNA is initially unwound by the helicase in the presence of ATP, and the primase then catalyses the formation of a short RNA segment called a primer, and the DNA polymerase finally catalyses DNA synthesis [31, 32]. The protein coded by ORF45 showed a high sequence similarity to the terminases of the *Salmonella* phages (SETP5, SETP7 and SETP12), with an E value of $7e-81$, strongly suggesting that ORF45 is the coding gene for the EEP terminase. The terminase complex, comprising one small and one large subunit [33], is a key component of the dsDNA packaging machine for packing replicated genomic DNA into preassembled empty capsid shells using its endonuclease and ATPase activities [34–36]. The coding product of ORF45 corresponded to the large subunit of EEP terminase. Many of the ORFs in this module might encode unique products which shared no homologies with proteins present in the microbial database, and we were not able to predict their function based on BLAST analysis. However, the known ORFs contained in EEP DNA replication and packaging modules show a high sequence similarity to the same proteins of *Salmonella* phages, e.g. *Salmonella* phage SETP3, KS7 and KS73.

Analogous to other dsDNA bacteriophages, the structural module in phage EEP is located next to the DNA replication module [37]. Some structural proteins of phage EEP were examined by SDS-PAGE (fig. 7), and N-terminal sequencing of 6 structural proteins was performed (table 3). Compared with the protein sequence of the 52 coding ORFs, only ORF21 coded a product (125.7 kDa) close to 123.5 kDa. The N-terminal sequences (Met-Ile-Thr-Thr-Tyr) for the 123.5-kDa protein in SDS-PAGE (fig. 7) matched with the predicted N-terminal sequence of ORF21, which showed sequence similarity with a tail fiber protein of enterobacterium phage HK97. The N-terminal sequences of the 90.2- and 26.3-kDa proteins isolated from SDS-PAGE were identified as Ala-Asp-Gln-Ala-Ala and Gly-Tyr-Gln-Leu-Pro, and matched with the predicted amino acids of ORF26 and ORF29, respectively. ORF26 and ORF29 shared high sequence similarity with the phage tail tape measure protein of *Shigella flexneri* 2a strain 301 and a major tail protein of enterobacterium phage T1, respectively. Similarly, the 65.5- and 15.6-kDa structural proteins were also determined to be coded by ORF46 and ORF40. The inability to identify the coding gene for the major EEP coat protein (the 40.5-kDa protein from SDS-PAGE) may possibly be explained by the inability to predict the corresponding ORF using bioinformatics. Otherwise, ORFs coding for putative phage tail proteins (ORF22, ORF23, ORF24, ORF25 and ORF52), phage head morphogenesis protein (ORF47) and

conserved phage structural proteins (ORF32 and ORF34) were also classified into the structural module of the EEP genome. Interestingly, the nucleotide sequences of ORFs in the EEP structural module were found to share a high degree of homology with the sequence of structural proteins of enterobacterial phages, e.g. N15, HK022, HK97, TLS and T1.

ORF39 belongs to the lysis module of phage EEP. Lysozyme is one of the most important functional proteins for bacteriophages and has the ability to hydrolyze the β 1– β 4 linkage of the peptidoglycan backbone of the bacterial cell wall [38]. The product coded by ORF39 showed homology with lysozyme with a score of 93.6, and was thus identified as lysozyme.

The product coded by ORF18 was homologous to the superinfection exclusion COR-like protein of the enterobacterial phage N15, with an E value of $2e-08$. This protein acts to prevent EEP from being substituted by other superinfecting phages.

In summary, we experimentally identified the coding genes for 5 constituent proteins, which, together with the 18 known-function genes predicted by bioinformatics searching, means that 23 genes of the EEP genome were functionally delineated in the present study.

The elucidation of the physicochemical and genomic properties of this novel coliphage, which has strong lytic ability to engineered *E. coli* strains, may help to elucidate phage diversity and to establish effective phage control strategies in fermentation industries and laboratory environments.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (30700711).

References

- 1 Lima-Mendez G, Toussaint A, Leplae R: Analysis of the phage sequence space: the benefit of structured information. *Virology* 2007;365:241–249.
- 2 Boyd EF, Brussow H: Common themes among bacteriophage-encoded virulence factors and diversity among the bacteriophages involved. *Trends Microbiol* 2002;10:521–529.
- 3 Frost LS, Leplae R, Summers AO, Toussaint A: Mobile genetic elements: the agents of open source evolution. *Nat Rev Microbiol* 2005;3:722–732.
- 4 Smets BF, Barkay T: Horizontal gene transfer: perspectives at a crossroads of scientific disciplines. *Nat Rev Microbiol* 2005;8:459–465.
- 5 Waldor MK, Friedman DI: Phage regulatory circuits and virulence gene expression. *Curr Opin Microbiol* 2005;8:459–465.
- 6 Hendrix RW: Bacteriophage genomics. *Curr Opin Microbiol* 2003;6:506–511.
- 7 Ackermann HW: 5500 phages examined in the electron microscope. *Arch Virol* 2007;152:227–243.
- 8 Iida Y, Matsuda Y, Saito R, Nakasato M, Nonomura T, Kakutani K, Tosa Y, Mayama S, et al: Efficient release of overproduced gene products from *Escherichia coli* BL21 (DE3) by lytic infection with newly isolated bacteriophages. *Biosci Biotechnol Biochem* 2003;67:198–202.
- 9 Govind R, Fralick JA, Rolfe RD: Genomic organization and molecular characterization of *Clostridium difficile* bacteriophage Φ CD119. *J Bacteriol* 2006;188:2568–2577.
- 10 Casas V, Rohwer F: Phage metagenomics. *Methods Enzymol* 2007;421:259–268.
- 11 Shelton CB, Crosslin DR, Casey JL, Ng S, Temple LM, Orndorff PE: Discovery, purification, and characterization of a temperate transducing bacteriophage for *Bordetella avium*. *J Bacteriol* 2000;182:6130–6136.
- 12 Lu Z, Breidt FJ, Fleming HP, Altermann E, Klaenhammer TR: Isolation and characterization of a *Lactobacillus plantarum* bacteriophage, Φ JL-1, from a cucumber fermentation. *Int J Food Microbiol* 2003;84:225–235.
- 13 Wu LT, Chang SY, Yen MR, Yang TC, Tseng YH: Characterization of extended-host-range pseudo-T-even bacteriophage Kpp95 isolated on *Klebsiella pneumoniae*. *Appl Environ Microbiol* 2007;73:2532–2540.
- 14 Capra ML, Quiberoni A, Reinheimer JA: Thermal and chemical resistance of *Lactobacillus casei* and *Lactobacillus paracasei* bacteriophages. *Lett Appl Microbiol* 2004;38:499–504.
- 15 Dong H, Lin W, Zhang CK, Xiong H, Fu G, Jin WR, Chen R, Chen Z, et al: Genomic sequence and expression analyses of human chromatin assembly factor 1 p150 gene. *Gene* 2001;264:187–196.
- 16 Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb JF, et al: Whole genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* 1995;269:496–512.
- 17 Istrail S, Sutton GG, Florea L, Halpern AL, Mobarry CM, Lippert R, Walenz B, Shatkay H, et al: Whole-genome shotgun assembly and comparison of human genome assemblies. *Proc Natl Acad Sci USA* 2004;101:1916–1921.
- 18 Blattner FR, Plunkett GIII, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, et al: The complete genome sequence of *Escherichia coli* K-12. *Science* 1997;277:1453–1462.
- 19 Ackermann HW, DuBow MS: Viruses of Prokaryotes, vol II: Natural Groups of Bacteriophages. Boca Raton, CRC Press, 1987, pp 1–54, 171–218.
- 20 Ackermann HW: Frequency of morphological phage descriptions in the year 2000. *Arch Virol* 2001;146:843–857.
- 21 Sechaud L, Cluzel PJ, Rousseau M, Baumgartner A, Accolas JP: Bacteriophage of lactobacilli. *Biochimie* 1988;70:401–410.
- 22 Ackermann HW: Tailed bacteriophage: the order caudovirales. *Adv Virus Res* 1998;51:135–201.
- 23 Ruokoranta TM, Grahm AM, Ravaniti JJ, Poranen MM, Bamford DH: Complete genome sequence of the broad host range single-stranded RNA phage PRR1 places it in the Levivirus genus with characteristics shared with Alloviruses. *J Virol* 2006;80:9326–9330.
- 24 Majumdar S, Barman S, Das J: Phage phi 92: a new cholera phage infecting *Vibrio cholerae* cells belonging to serovars 01 and non-01. *Intervirology* 1993;36:237–244.

- 25 Synnott AJ, Kuang Y, Kurimoto M, Yamamichi K, Iwano H, Tanji Y: Isolation from sewage influent and characterization of novel *Staphylococcus aureus* bacteriophages with wide host ranges and potent lytic capabilities. *Appl Environ Microbiol* 2009;75: 4483–4490.
- 26 Capra ML, Del L, Quiberoni A, Ackermann HW, Moineau S, Reinheimer JA: Characterization of a new virulent phage (MLC-A) of *Lactobacillus paracasei*. *J Dairy Sci* 2006;89: 2414–2423.
- 27 Bryner JH, Ritchie AE, Foley JW, Berman DT: Isolation and characterization of a bacteriophage for *Vibrio fetus*. *J Virol* 1970;6: 94–99.
- 28 Musarrat J, Ahmad M: Damage and mutagenesis of bacteriophage lambda induced by high pH. *Mutagenesis* 1991;6:207–211.
- 29 Overcast WW, Nelson FE, Parmelee CE: Influence of pH on proliferation of lactic *Streptococcus* bacteriophage. *J Bacteriol* 1951;61: 87–95.
- 30 Brussow H, Hendrix RW: Phage genomics: small is beautiful. *Cell* 2002;108:13–16.
- 31 Lee J, Chastain PD, Griffith JD, Richardson CC: Lagging strand synthesis in coordinated DNA synthesis by bacteriophage t7 replication proteins. *J Mol Biol* 2002;316:19–34.
- 32 VanLoock MS, Chen YJ, Yu X, Patel SS, Egelman EH: The primase active site is on the outside of the hexameric bacteriophage T7 gene 4 helicase-primase ring. *J Mol Biol* 2001; 311:951–956.
- 33 Kanamaru S, Kondabagil K, Rossmann MG, Rao VB: The functional domains of bacteriophage t4 terminase. *J Biol Chem* 2004;279: 40795–40801.
- 34 Alam TI, Rao VB: The ATPase domain of the large terminase protein, gp17, from bacteriophage T4 binds DNA: implications to the DNA packaging mechanism. *J Mol Biol* 2008;376:1272–1281.
- 35 Maluf NK, Gaussier H, Bogner E, Feiss M, Catalano CE: Assembly of bacteriophage lambda terminase into a viral DNA maturation and packaging machine. *Biochemistry* 2006;45:15259–15268.
- 36 Ponchon L, Boulanger P, Labesse G, Letellier L: The endonuclease domain of bacteriophage terminases belongs to the resolvase/integrase/ribonuclease H superfamily: a bioinformatics analysis validated by a functional study on bacteriophage T5. *J Biol Chem* 2006;281:5829–5836.
- 37 Zimmer M, Scherer S, Loessner MJ: Genomic analysis of *Clostridium perfringens* bacteriophage ϕ 3626, which integrates into *guaA* and possibly affects sporulation. *J Bacteriol* 2002;184:4359–4368.
- 38 Rossmann MG, Mesyanzhinov VV, Arisaka F, Leiman PG: The bacteriophage T4 DNA injection machine. *Curr Opin Struct Biol* 2004;14:171–180.