



RESEARCH ARTICLE

Characterization and Genome Analysis of a Novel *Escherichia coli* Bacteriophage vB_EcoS_W011D

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ABSTRACT

The application of phages against the increased reported drug resistant *Escherichia coli* is one of the promising alternative therapeutic options. The total number of phages on the earth are more than 10^{31} , but the phages that have been isolated and studied are limited. Hence, discovering of new phage and uncovering its characteristics will provide materials for extensive use of phage therapy in the future. In this study, a novel *E. coli* phage named vB_EcoS_W011D was isolated and the characteristics and genome were explored. The typical morphology of vB_EcoS_W011D is comprised of an *icosahedral* head and a constricted flexible rolled up tail, revealing that it is the genus TLS virus of Tunavirinae subfamily. One-step growth curve showing the eclipse and latent period of vB_EcoS_W011D was 5 min and 10 min, respectively, with the burst size of 115 PFU/cell. The genome of vB_EcoS_W011D is double-stranded consisting of 49,847 bp with 46.24% of G+C contents and shows $\leq 77\%$ similarities (with 38% query coverage) to other reported phages. A total 85 putative ORFs were identified. Of which, 43 predicted ORFs had significant homology with other phage proteins of known functions. A putative Zonula occludens toxin was found in its genome. In addition, a clear difference was revealed on the phylogenetic analysis of it terminates large subunit and capsid protein. In conclusion, our study clearly indicates that vB_EcoS_W011D is a newly discovered *E. coli* phage that could be further investigated to elucidate phage variety and evolutionary relationship between bacteria and phages.

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INTRODUCTION

Escherichia coli is a widespread bacterium in the intestines of warm-blooded animals and associated with variety animal and human diseases (Jang *et al.*, 2017; Dusek *et al.*, 2018). The infection caused by *E. coli* can be fatal, especially for young and elderly human and animals. However, the control of *E. coli* infection has become more difficult mainly due to the emergency of antibiotic-resistant bacterial strains and deficiency of novel effective antibiotics (Aslam *et al.*, 2018; Bloom *et al.*, 2018).

Bacteriophages (phages) were thought to be a promising alternative strategy for controlling of bacterial infections, especially for multidrug-resistant bacteria. Phage shows no harmful to animals and human (Moelling, 2018). There are so many reports about successfully application of phage in controlling the animal diseases caused by *E. coli* (Valério *et al.*, 2017; Manohar *et al.*, 2019). Additionally, phages are indispensable in regulating global biochemical cycle and play important role as models for molecular biology studies to explore the basic cellular processes (Casjens *et al.*, 2015). Moreover, phage have also been widely used in genetic engineering and biotechnology to construct newly

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recombinant phage to control diseases caused by bacterial infection among animals (Stanley, 2018; Chen *et al.*, 2019).

So far, there are many *E. coli* phages have been reported, but the knowledge about their diversity and function is relatively poor due to the huge amount and existent almost everywhere on the earth. In addition, even phages isolated using the same host have different genomes and characteristics (Doss *et al.*, 2017). Therefore, the discovery of new phages and exploring basic features and genomic diversities among phage species are necessary for exploring the evolutionary relationship between bacteria and phages. In addition, it can provide candidate material for phage application in the future. Additionally, bacteria and phages are constantly evolving in the process of confrontation, so discovering of new phage is the key to timely control bacteria. In this study, a novel *E. coli* phage vB_EcoS_W011D was isolated from sewage. The characteristics and genome of this phage have been studied.

MATERIALS AND METHODS

Bacterial strain and growth conditions: *Escherichia coli* 011D was isolated from clinical animal samples using repeated plate streaking on Lauria Broth solid-medium with 1.5% agar. The purified colony was identified by biochemical tests (VITEK2 Compact, France bio,) and confirmed by sequence analysis of conserved segment of 16SrRNA gene with universal primers F (5'-TCAACC GGGGAGGGT-3') and R (5'-TCAACCGGGGAGGGT-3'). The purified strain was stored in LB containing 30% glycerol at -20°C and -80°C, respectively.

Isolation and purification of vB_EcoS_W011D phage:

The phage isolation process was performed as previous description (Gu *et al.*, 2012). The whole procedure was repeated three times to get the purified vB_EcoS_W011D phages. Then purified phages were stored at 4°C or mixed with 30% glycerol in LB and stored at -80°C.

TEM observation of vB_EcoS_W011D: The purified phages were applied to 200 mesh copper grids and negatively stained with phosphotungstic acid (2%, w/v). The transmission electron microscopy (HEOL JEM-1200EXII; Japan Electronics and Optics Laboratory, Tokyo, Japan) was used to examine morphology at accelerating voltage of 80 kV.

One-Step Growth Curve Analysis of vB_EcoS_W011D:

Intracellular lytic process of vB_EcoS_W011D was detected by one-step growth experiment with multiplicity of infection (MOI) of 0.1 (Gu *et al.*, 2012). Two sets of samples were collected every 5 min for the first 20 min, then collection was performed every 10 min until 60 min. One set of collection were pre-treated with 1% (v/v) chloroform for 30 min (Saralamba *et al.*, 2018), and another set of collection were treated with nothing. Then double layer agar method was performed to estimate the titer of phage at different stages of one-step growth. The burst size was estimated as the ratio of final phage

number which was counted at end of one cycle of growth to the number of infected bacteria (Xi *et al.*, 2019). The procedure was repeated three times.

Genome Sequencing and Bioinformatics Analysis of vB_EcoS_W011D:

Genomic DNA of vB_EcoS_W011D was extracted using a viral genome extraction kit (Omega B IO-Tek Inc., Doraville, GA, USA) according to guidelines of manufacturer. The extracted genome was sequenced by Wuhan Genomics Institute using an Illumina Hiseq system. SPAdes v.3.6.2 was used to assemble sequences. GeneMarkS v.3.6.2 was used to predict potential ORFs, and ORFs were verified using Rapid Annotation using Subsystem Technology, version 2.0 (RAST) annotation server (Aziz *et al.*, 2008). BLAST analysis available at NCBI website and HMMER software were used to predict the ORFs (Altschul *et al.*, 1997). Possible tRNAs were predicted by tRNA scanner (<http://lowelab.ucsc.edu/tRNAscan-SE/>). CLC Genomics Workbench 6.8 (CLC Bio-Qiagen, Aarhus, Denmark) was used to visualize all function-related modules. CG View (http://stothard.afns.ualberta.ca/cgview_server/index.html) was employed to perform GC skew and content.

Evolutionary relationship Analysis of vB_EcoS_W011D:

The phylogenetic tree was constructed based on terminase large subunits and capsid proteins, respectively (Altschul *et al.*, 1997; Aziz *et al.*, 2008). Briefly, the top fifty highly-homology nucleic acid sequence from different phages were obtained. then the neighbor joining phylogenetic tree of the major capsid protein and terminase large subunit were made by MEGA5.05 with 1000 bootstrap replicates.

RESULTS

Biological features of vB_EcoS_W011D: The vB_EcoS_W011D was isolated from sewage using *E. coli* 011D as host strain. Purified vB_EcoS_W011D formed transparent spots on *E. coli* 011D lawn (Fig. 1A). Electron microscopy showed that the particle of vB_EcoS_W011D was mainly comprised of two parts, a head with a diameter of 46±5 nm (n=3) and rolled up tails with length of 117±5 nm (n=3), which does not match to most of the other reported *E. coli* phage. Therefore, we conclude that the vB_EcoS_W011D belongs to genus TLS virus of Tunavirinae subfamily (Fig. 1B).

As shown in Figure 1C, one-step growth curve of vB_EcoS_W011D showed that the eclipse and latent period of vB_EcoS_W011D was 5 min and 10 min, respectively. It could totally lyse host strain within 30 minutes with a burst size of 115 PFU per cell.

Genome Characteristics of the vB_EcoS_W011D:

The genome sequencing indicated that vB_EcoS_W011D is a double-stranded DNA virus with genome comprised of 49,847 bp, with an average 46.24% G+C contents (Figure 2). No tRNAs was found in the genome, meaning that this phage might rely on tRNAs of host cell to express functional genes. BLAST analysis of whole genome against existing phage genome in database revealed that vB_EcoS_W011D shows 77.51% homologous to the *Shigella* phage pSf-1 (Accession number: KC710998.1),

75.76-76.04% related to the *Citrobacter* phages (Accession number: MH729819.1; KM236241.1; KY694971.1), and 75-76% similar to the *Salmonella* phages (Accession number: MG241338.1; KY657202.1; KX015771.1). In addition, vB_EcoS_W011D shows $\leq 75.67\%$ identity to other *Escherichia* phages, namely, vB_EcoS-95 (Accession number: MF564201.1), vB_Eco_swan01 (Accession number: LT841304.1), LL5 and SECphi27 (Accession number: MH491968.1 and LT961732.1).

Genome analysis of vB_EcoS_W011D indicated a total of 85 predicted open reading frames (ORFs). CLC Genomics Workbench 6.8 was used to visualize all function-related modules. As shown in Fig. 3, a total of 38 ORFs were predicted to be function-related, which were mainly related to phage morphology, nucleotide metabolism and replication, and lysis system. In addition, no lysogeny modules, antibiotic-resistant genes, or putative virulence factors were found in the predicted results.

Morphology module: There were twenty-three ORFs of vB_EcoS_W011D encoding structural proteins including head protein (ORF2, ORF3, ORF5-7, ORF12-15), and tail protein (ORF74-83). Both of these proteins are similar ($\geq 80\%$) to phages which originate from *Escherichia*, *Salmonella* and *Enterobacteria* phages. Remarkably, ORF4 was located in morphology module that encode for a putative zonula occluded toxin (Zot), which presents 58.23% similarity to that of *Salmonella* phage 36 (Accession number: KR296690.1).

Nucleotide metabolism and replication-related module: ORFs encoding phage replication-associated proteins including ATP-dependent helicase (ORF64), DNA primase (ORF66), single-stranded DNA binding protein Ssb (ORF68) and exodeoxyribonuclease VIII (ORF70) were identified. ORF64 showed 91% homology to DNA helicase of *Escherichia* virus vB_Eco_mar001J1 (Accession number: LR027388.1). ORF66 had 86% identity to DNA primase of *Escherichia* phage vB_Eco-95 (Accession number: MF564201.1). ORF68 was similar to single-stranded DNA binding protein Ssb of *Escherichia* virus vB_Eco_AKS96 with identity of 75.6%. ORF70 was homologous to exodeoxyribonuclease VIII of *Escherichia* phage LL5 with 92% identity (Accession number: MH491968.1). Based on these findings, we hypothesize that the replication of vB_Eco_W011D might be dependent on nucleotide excision repair pathway.

ORF24, ORF27, ORF34, ORF44-46, ORF49 and ORF57 in the vB_Eco_W011D genome associated with nucleotide metabolism. ORF24 encoded an ATP-binding protein which was found in combination of Walker A motif and universal stress proteins that could provide energy to drive biochemical reaction in the cells (Bustamante *et al.*, 2004). ORF27 encoded an acid phosphatase showed 85% identity to that of *Chlamydia trachomatis*. ORF34, ORF44-46, and ORF57 are involved in the hydrolysis of ATP to ADP. Interestingly, ORF49 encoded a helicase, and which was found highly homologous to that of *Escherichia* phage LL5, was however not located in front of lysis module.

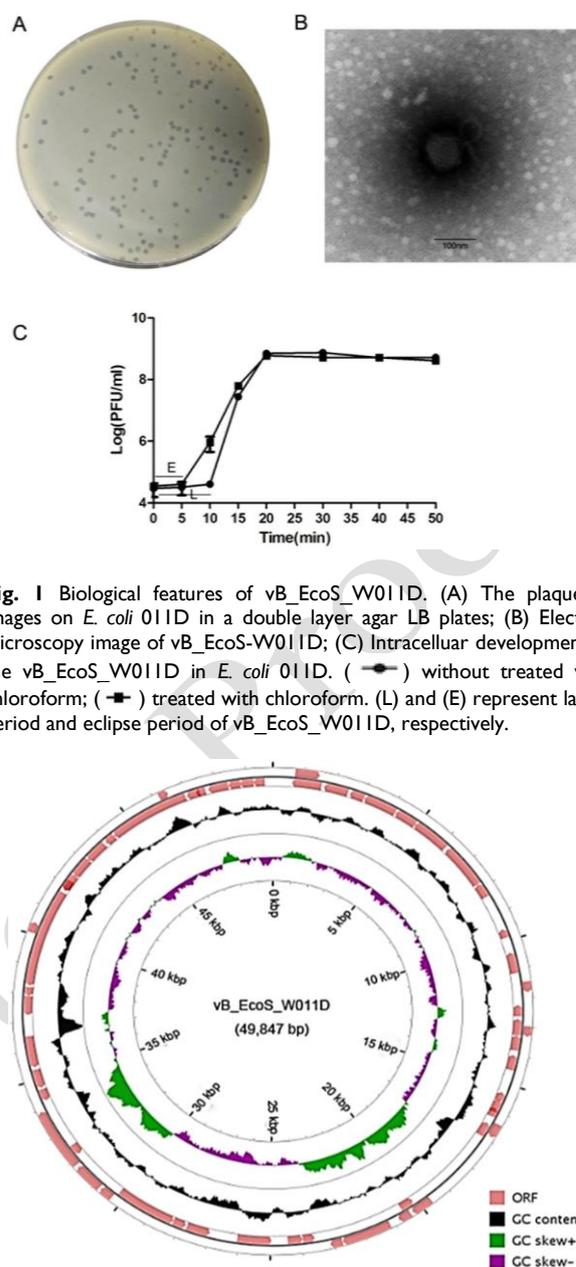


Fig. 1 Biological features of vB_EcoS_W011D. (A) The plaque of phages on *E. coli* 011D in a double layer agar LB plates; (B) Electron microscopy image of vB_EcoS-W011D; (C) Intracellular development of the vB_EcoS_W011D in *E. coli* 011D. (○) without treated with chloroform; (■) treated with chloroform. (L) and (E) represent latent period and eclipse period of vB_EcoS_W011D, respectively.

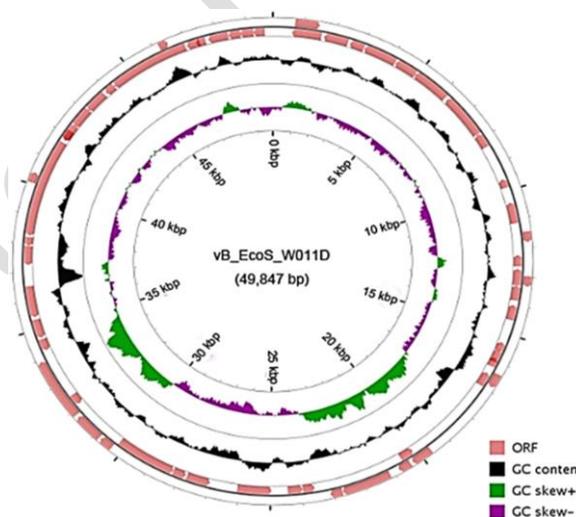


Fig. 2 Circle map of vB_EcoS_W011D genome. CG View server was used to develop the map. The physical organization was scaled in Kb, the transcription direction of ORFs was displayed by arrows, GC content and GC skew were shown in different color

Of note, ORF8 (terminase large subunit) and ORF9 (putative terminase small subunit) were highly conservative and that could potentially recognize tip of capsid protein and specific packaging site by hydrolyzing ATP. Both of them were found highly-related to those of *Escherichia* virus vB_Eco_mar001J1 (92% identity) and *Escherichia* phage vB_Eco_swan01 (82% identity). ORF65, encoded a putative transcriptional regulator, showing 85.5% similarity to *Escherichia* virus vB_Eco_mar001J1.

Lysis module: Lytic section of vB_Eco_W011D consist of ORF53, ORF54 and ORF55. ORF53 encoded a putative unimolecular spanin, which showed 67% homology to that of *Citrobacter* virus Stevie (Accession number: YP_009148746.1). ORF54 and ORF55 encoded a putative endolysin and a hypothetical holin, respectively.

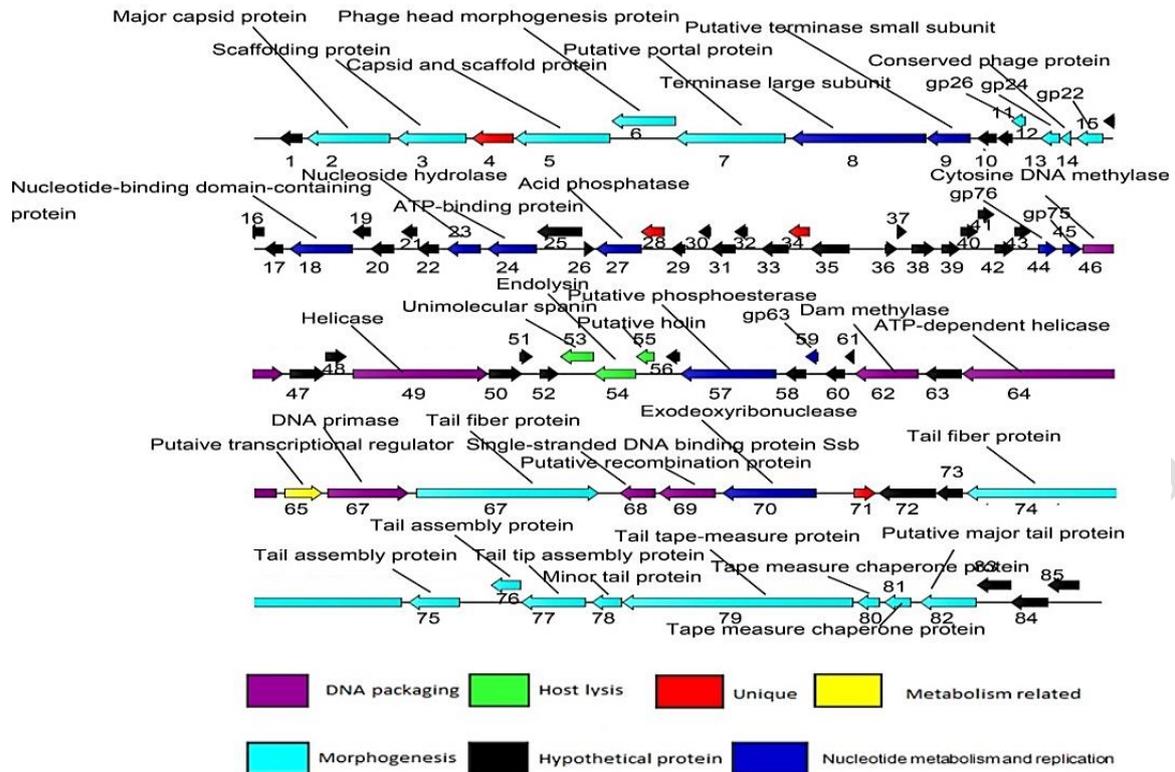


Fig. 3: Graphical representation of vB_EcoS_W011D genome. There are 85 ORFs predicted and performed for bioinformatics analysis to assess their function. The direction of transcription was shown by arrows. The genome map was drawn using CLC workbench 6.8.

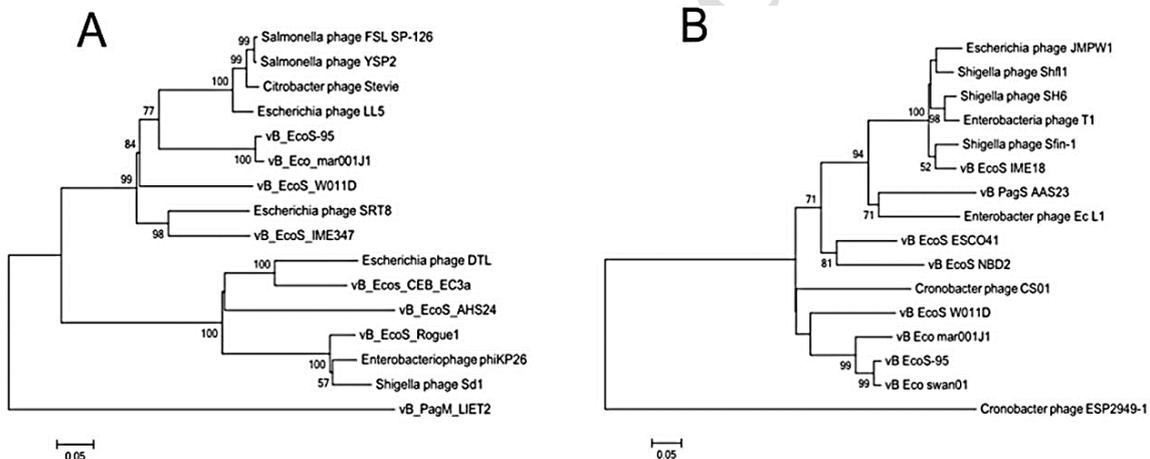


Fig. 4: A, B The neighbor joining phylogenetic tree of the major capsid protein (A) and terminase large subunit (B). Trees were made by MEGA 5.05 with 1000 bootstrap replicates.

Notably, ORF71 is encoded for a putative superinfection exclusive (Sie) protein, which is located in the lysis module homologous to *Escherichia* phage JMPW2 (52%/73%) (Accession number: ALT58170.2), that was shown associated with host protect mechanism against other invading phages in the process of phage replication.

Evolutionary relationship of vB_EcoS_W011D: *Cronobacter* phage ESP2949-1TLU and *Pantoea* phage vB_PagM_LIET2 were selected as out group candidates, respectively, to analysis the most closely related relationship of vB_EcoS_W011D to other phages. In Figure 4A, *Escherichia* phage vB_EcoS-95 and *Escherichia* virus vB_Eco_mar001J1 are sister clade of capsid proteins of vB_EcoS_W011D but presents less than 84% identity. As shown in Figure 4B, terminase

large subunits of vB_EcoS_W011D shows similarity to *Escherichia* phage YSP2, *Escherichia* phage LL5, *Salmonella* phage FSL SP-126, *Salmonella* phage Stevie, *Escherichia* virus vB_Eco_mar001J1, *Escherichia* phage vB_Eco_swan01 and *Escherichia* phage vB_EcoS-95 with less than 80% identity. Altogether, the current data clearly demonstrated that vB_EcoS-W011D is obviously a novel branch of *Escherichia* phage that might deeply be used to uncover the role of phage in bacterial evolution.

Nucleotide sequence accession numbers: The accession number of the 16S sequence of *E. coli* 011D is MN015021 in the GenBank database. The accession numbers of vB_EcoS_W011D is MK77845 in the GenBank database. The accession number of the raw fastq files is SRS4580580 in the GenBank database.

Table S1: Summary of nucleic acid information of major capsid protein and terminase large subunit mentioned in this paper

Phage name (Major capsid protein)	Accession number	Phage name (Terminase large subunit)	Accession number
Salmonella phage FSL SP-126	KC139513.1	Escherichia virus vB_Eco_mar001J1	LR027385.1
Salmonella phage YSP2	MG241338.1	Escherichia phage vB_EcoS-95	MF564201.1
Citrobacter phage Stevie	NC_027350.1	Escherichia phage vB_Eco_swan01	LT841304.1
Escherichia phage LL5	MH491968.1	Cronobacter phage ESP2949-1	NC_019509.1
Escherichia phage vB_EcoS-95	MF564201.1	Cronobacter phage CS01	MH845412.1
Escherichia virus vB_Eco_mar001J1	LR027385.1	Escherichia phage vB_EcoS_ESCO41	KY619305.1
Pantoea phage vB_PagM LIET2	MK388689.1	Enterobacteria phage vB_EcoS_NBD2	NC_031050.1
Escherichia phage SRT8	MF996376.1	Pantoea phage vB_PagS_AAS23	MK095606.1
Enterobacteria phage vB_EcoS_IME347	MH051918.1	Shigella phage Sfin-1	MF468274.1
Escherichia phage vB_EcoS_AHS24	NC_024784.1	Escherichia phage JMPW1	KUI94206.1
Escherichia phage DTL	MG050172.1	Shigella phage SH6	KX828710.1
Escherichia phage vB_Ecos_CEB_EC3a	KY398841.1	Escherichia virus T1	NC_005833.1
Enterobacteriophage phiKP26	KC579452.1	Enterobacteria phage vB_EcoS_IME18	MH051911.1
Shigella phage Sdl	MF158042.1	Enterobacter phage Ec_L1	MG732930.1
Enterobacteria phage vB_EcoS_Rogue1	NC_019718.1	Shigella phage Shf11	NC_015456.1

DISCUSSION

In this study, a new bacteriophage named vB_EcoS_W011D has been isolated and characterized. One of the remarkable features of vB_EcoS_W011D is the presence of a non-typical lysis protein, the unimolecular spanin (ORF53) which has been shown involved in the destruction of the outer membrane of Gram-negatives at the final stage of host lysis (Kongari *et al.*, 2018). Furthermore, endolysin (ORF54) and a holin (ORF55), the typical lysis proteins, are also included in the lysis section of vB_EcoS_W011D, which indicating the holin-endolysin lysis system of vB_EcoS_W011D was employed to kill bacteria. At first, with the help of holin, the inner membrane was destroyed to form micron-scale holes, then the actively lysin was releasing to degrade the peptidoglycan (Cahill, 2019). According to recent study, membrane disrupting chemicals such as spanin can enhance the efficiency of endolysin (Han *et al.*, 2014). It is therefore hypothesized here that the endolysin spanin (ORF53) of vB_EcoS_W011D would facilitate lysis of host membrane during the invasion as reported previously for other endolysins (Kong *et al.*, 2015).

The intriguing property of vB_EcoS_W011D is the presence of a novel zonula occludens toxin (Zot) gene encoded by its genome, which mainly encoded by filamentous phages and it has been shown to be involved in tail morphology of phages (Waldor *et al.*, 1996, Castillo *et al.*, 2018). Considering the extremely crooked vB_EcoS_W011D, we hypothesize that it could be a reason for extremely rolled up tails. Additionally, it has been shown that Zot could change the tight junction of epithelial cells and contribute to increase the paracellular transport of macromolecules, in a non-toxin manner, and it can be employed as new-type of vaccine vehicle also (Ruane *et al.*, 2013).

Notably, a putative super-infection exclusive (Sie) protein has been found in its genome, the same functional protein has been found in Bacteriophage HK97 (Cumby *et al.*, 2012), Citrus tristeza virus (Dawson *et al.*, 2015), Salmonella phage P22 (Zinno *et al.*, 2014) and Temperate Streptococcus thermophilus phage TP-J34 (Ali *et al.*, 2014) in recent years. As is the case with Sie of Citrus tristeza virus requires production of a specific viral protein. Moreover, the Sie of Salmonella phage P22 was demonstrated involve in abortive infection, which could lead to death of a cell infected by two phages before the Sie contain phage produce progeny (Zinno *et al.*, 2014)

and provide an evidence Sie might contribute to protect the lysogenized host from death by same of similar phages or other bacteriocins.

Both of Zot and Sie were primarily found in bacteria, and its existence in the genome of vB_EcoS_W011D indicate horizontal gene transfer which have been confirmed was involved in co-evolution of bacteria and phage. Furthermore, it can be benefit for phage to reduce the chances of entering lysogenization (Frazão *et al.*, 2019). More importantly, few functional genes of vB_EcoS_W011D, such as chaperone protein and tail assembly protein which were highly similar to that of other bacteria *Salmonella* phages and *Citrobacter* phage, respectively, is indicative of evolutionary process.

Conclusions: a novel lytic phage vB_EcoS_W011D against *E. coli* 011D was isolated and characterized. However, our data show that vB_EcoS_W011D displayed a rapid and strong cell lysis pattern and is comprised of two unique genes putatively encoded for Zot and Sie proteins that could further be studied to reveal the variety of bacteriophage and their evolutionary relationship with bacteria.

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Author contributions: WYH, JMG and JBD conceived and designed the study; XWW, HYX, JZS and ZJW contributed to the writing and revision of the manuscript; XWW, HYX, JZS, DLH, MJC, RPC performed laboratory testing; XWW, HYX and JZS contributed to the genome sequencing and analysis. CJS and SUR read and revised the manuscript. All authors read and approved the final manuscript.

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