



A *Siphoviridae* bacteriophage specific to extended-spectrum β -lactamases-producing *Escherichia coli*

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ABSTRACT

Extended-spectrum β -lactamases-producing *Escherichia coli* (ESBL-producing *E. coli*) pose a threat to antibiotic based therapeutic approach because of their multidrug resistance properties. The bacteriophage therapy has recently become a promising alternative therapeutic approach. The aims of this study are to isolate and classify a bacteriophage specific to ESBL-producing *E. coli* and to examine its host range. In this study, a bacteriophage, ϕ UBU-ESBL, was isolated from water collected from a wastewater treatment pond. It was found to produce small clear plaques of 1-2 mm in diameter and to inhibit only *E. coli*-ESBL, but not other bacteria used in this study. Its genome was digested by the restriction enzyme BamHI indicating that the genome was double stranded DNA. As revealed by transmission electron microscopy, ϕ UBU-ESBL had an isometric head (50 ± 3.4 nm in diameter) with a noncontractile tail (290 ± 15.1 in length). Based on its genomic and morphological characteristics, ϕ UBU-ESBL was classified as a member in the family *Siphoviridae*. This study provides preliminary information showing that ϕ UBU-ESBL had potential for further study towards its application as a therapeutic agent against ESBL-producing *E. coli* infectious diseases.

Keywords: Bacteriophage, *Escherichia coli*, extended-spectrum β -lactamases

INTRODUCTION

Extended-spectrum β -lactamases-producing *Escherichia coli* (ESBL-producing *E. coli*) are antibiotic resistant strains of *E. coli* having ability to manufacture an enzyme called extended-spectrum β -lactamases. Currently, they have caused a global health problem because they are emerging worldwide and resistant to many antibiotics. They are resistant to all penicillins, to cephalosporins (including third and fourth generation agents), and to aztreonam. Furthermore, they are often cross-resistant to trimethoprim/sulfamethoxazole and quinolones [1]. ESBL-producing *E. coli* can cause a wide range of infections, ranging from urinary tract infections to severe blood poisoning [1, 2]. Infections with ESBL-producing *E. coli* most commonly occur in the elderly, people who have recently been in hospitals, and people who receive or have received antibiotic treatment. Since most antibiotics are ineffective in treating ESBL-producing *E. coli* infections, alternative antimicrobial agents against the bacteria are required.

The emergence of ESBL-producing *E. coli* has urged scientists to search for alternative therapeutic approaches to combat infectious diseases caused by the problematic bacteria. One of the potential candidates is bacteriophage therapy (or phage therapy). This therapeutic approach uses bacteriophages, bacterial viruses, as antimicrobial agents to inhibit bacterial growth or to kill bacteria [3]. Since the discovery by Twort in 1915 and by d'Hérelle in 1917, bacteriophages have been used for treatment and prophylaxis of various bacterial infectious diseases. Therapeutic phages have been reported to have advantages over antibiotics [4]. Chances of developing serious side effects by bacteriophages are miniscule because of their high host specificity. Moreover, small doses of bacteriophages can be used effectively because they are self-replicating in their target bacterial cells. Several reports have shown the ability of bacteriophages to kill drug resistant bacteria such as *Staphylococcus aureus* [5], *Klebsiella pneumoniae* [6],

Streptococcus pneumoniae [7], *Pseudomonas aeruginosa* [8] and *Vibrio parahaemolyticus* [10]. Therefore, it is of interest to find a bacteriophage for use as a therapeutic agent to control ESBL-producing *E. coli*.

This study aims to isolate a bacteriophage specific to ESBL-producing *E. coli* from wastewater and to partially characterize the bacteriophage in some aspects such as host range, morphology and genome. The bacteriophage from this study may be useful as a potential therapeutic agent for controlling ESBL-producing *E. coli* infections.

EXPERIMENTAL SECTION

Bacterial strains and culture conditions

ESBL-producing *E. coli* used in this study was kindly gifted by Sappasitthiprasong Hospital, Ubon Ratchathani, Thailand. It is a clinical strain isolated from a patient suffering from its infection. It was used as the host for bacteriophage isolation. The bacterial strains listed in Table 1 were used in bacteriophage host range determination. All of the bacteria were cultured in Brain Heart Infusion broth (BHI broth) at 37°C and kept as glycerol (20% v/v) stock at -20°C until use.

Bacteriophage isolation

Bacteriophage isolation was conducted according to the protocol previously described by Phumkhachorn and Rattanachaikunsopon [10]. A total of 18 wastewater samples used for bacteriophage isolation were collected from various sources such as animal farms, houses and wastewater treatment ponds in hospitals and industries in Ubon Ratchathani, Thailand. Ten ml of each sample was centrifuged at 3,500 rpm for 10 min. The supernatant was filtered through a 0.45 µm-pore-size membrane filter (SartoriusAG, Goettingen, Germany). Five ml of the filtrate was added to an equal volume of double strength BHI broth. One hundred µl of ESBL-producing *E. coli* overnight culture was added to the mixture and incubated at 37°C for 24 h. After incubation, the culture was centrifuged at 3,500 rpm for 10 min and the supernatant was filtered through a 0.45 µm-pore-size membrane filter. The resulting filtrate was used for examining the presence of bacteriophage by spot test method.

Spot test method

Spot test method was performed for detection of bacteriophage in the prepared filtrate samples. In brief, 100 µl of a log phase ESBL-producing *E. coli* culture was added to 5 ml of pre-warmed (60°C) soft agar (0.4% agar), gently mixed, and overlaid onto a BHI agar plate. After allowing the soft agar to solidify for 20 min, 10 µl of each prepared filtrate sample was spotted onto top agar layer. The spotted sample was allowed to dry and the plate was incubated at 37°C for 24 h before observing the presence of a clear zone. A clear zone at the spot area, representing the lysis of host cells, indicates the activity of bacteriophage. The filtrate sample giving a positive result was subjected to plaque assay to confirm the presence of a bacteriophage in the sample.

Plaque assay

The filtrate sample giving a positive result from the bacteriophage isolation experiment was subjected to ten-fold serial dilution using BHI broth as a diluent. One hundred µl of the suitable dilution and 100 µl of log phase ESBL-producing *E. coli* culture were added into 5 ml of pre-warmed (60°C) soft agar (0.4% agar) and mixed well. The mixture was poured onto the surface of a BHI agar plate. The plate was incubated at 37°C for 24 h to allow plaques to form. The formation of plaques indicates the presence of a bacteriophage in the filtrate sample. The number of plaques was used to calculate the bacteriophage titer expressed in plaque forming unit (PFU)/ml.

Bacteriophage purification

After plaque assay, a single plaque was picked and added into phosphate buffered saline (pH 7.0) followed by dilution and re-plating for two rounds of further single plaque isolation. The final single plaque isolate was picked and transferred into a tube containing 10 ml of log phase ESBL-producing *E. coli* culture in BHI broth. The tube was then incubated at 37°C for 24 h to allow bacterial cell lysis to occur. The bacteriophage lysate was centrifuged at 3,500 rpm for 10 min. The supernatant was filtrated through 0.45 µm-pore-size membrane filter. The resulting filtrate containing purified bacteriophage, called bacteriophage suspension, was kept at 4°C.

Bacteriophage host range determination

The spot test method (as described above) was used to determine the bacteriophage host range by using 10 bacterial strains listed in Table 1 as tested hosts.

Analysis of bacteriophage genome

Bacteriophage genome was extracted by using PureLink Viral RNA/DNA Mini Kit (Invitrogen, Carlsbad, CA, USA). The purified genome was tested for its sensitivity to the restriction enzyme *Bam*HI, RNase A and nuclease S1 (Sigma-Aldrich, St. Louis, MO, USA). The results were analyzed by 1% agarose gel electrophoresis.

Examination of bacteriophage morphology

The bacteriophage morphology was examined by transmission electron microscopy using the protocol previously described by Somnate et al. [11] with some modifications. A 10 μ l aliquote of purified bacteriophage suspension was spotted on top of a Formvarcoated copper grid (Proscitech, Brisbane, Queensland, Australia) and allowed to adsorb for 3 min at room temperature. The bacteriophage was stained by 2% uranyl acetate. The stained bacteriophage was examined by JEOL JEM-1230 Electron microscope (JEOL, Tokyo, Japan) at 80 kV accelerating voltage. The bacteriophage size was determined from the average of five independent measurements.

RESULTS

Bacteriophage isolation

Eighteen wastewater samples were used to screen for a bacteriophage specific to ESBL-producing *E. coli*. By using spot test method, only one sample (collected from a hospital's wastewater treatment pond) gave a positive result producing an inhibition zone on the lawn of ESBL-producing *E. coli* (Figure 1a). When the filtrate prepared from the sample was subjected to plaque assay using ESBL-producing *E. coli* as a host, it produced clear plaques of 0.1-0.2 mm in diameter, indicating the presence of a bacteriophage in the filtrate (Figure 1b). The bacteriophage were designated ϕ UBU-ESBL.

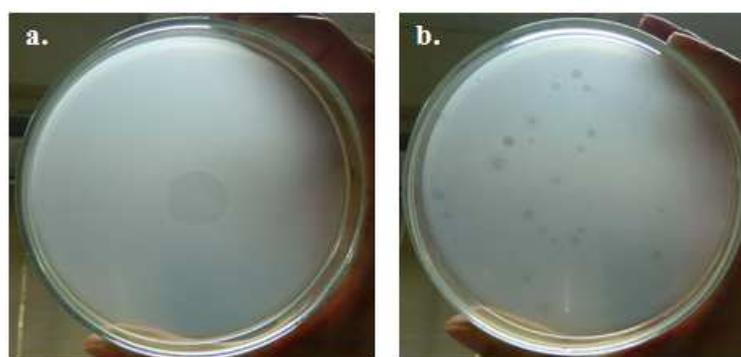


Figure 1: An inhibition zone (a) and plaques (b) on the lawn of ESBL-producing *E. coli* produced by ϕ UBU-ESBL

Bacteriophage host range determination

Host range of ϕ UBU-ESBL was determined by testing its lytic activity against the bacterial strains listed in Table 1. The bacteriophage had no lytic activity against all of the tested bacteria, suggesting that the bacteriophage was highly specific to ESBL-producing *E. coli*.

Table 1: Lytic activity of ϕ UBU-ESBL against different bacterial host strains

Bacterial host strain ^a	Lytic activity ^b
<i>Acinetobacter baumannii</i> (Drug resistant strain)	-
<i>Enterobacter aerogenes</i> (Drug resistant strain)	-
<i>Escherichia coli</i> ATCC25922	-
<i>Escherichia coli</i> 0157:H7 ATCC35150	-
<i>Klebsiella pneumoniae</i> ATCC27736	-
<i>Klebsiella pneumoniae</i> (Drug resistant strain)	-
<i>Pseudomonas aeruginosa</i> ATCC27853	-
<i>Salmonella enteritica</i> serovar Typhi DMST5784	-
<i>Shigella dysenteriae</i> ATCC13313	-
<i>Vibrio cholerae</i> O1, non O139 DMST2873	-

^a ATCC = American Type Culture Collection; DMST = Department of Medical Sciences Thailand; all drug resistant strains gifted by Sappasitthiprasong Hospital, Thailand

^b + = have lytic activity; - = have no lytic activity

Analysis of bacteriophage genome

Sensitivity to nucleic acid digesting enzymes of the genome extracted from ϕ UBU-ESBL was studied. The result demonstrated that the genome was digested by the restriction enzyme *Bam*HI (Figure 2). However, it was not digested by RNase A and nuclease S1.

Examination of bacteriophage morphology

Analysis of bacteriophage morphology by transmission electron microscopy revealed that ϕ UBU-ESBL was a tailed bacteriophage. It had an isometric head (50 ± 3.4 nm in diameter) with a noncontractile tail (290 ± 15.1 in length) (Figure 3). No collar, base plate and tail fiber were observed.



Figure 2: Restriction enzyme digestion analysis of ϕ UBU-ESBL genome. Lane 1, Lambda DNA digested with *Hind*III (DNA marker); lane 2, uncut ϕ UBU-ESBL genome; lane 3, ϕ UBU-ESBL genome cut with *Bam*HI

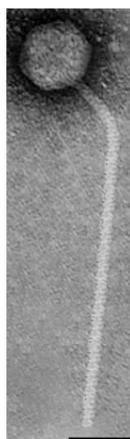


Figure 3: Morphology of ϕ UBU-ESBL genome as studied by transmission electron microscopy. (Bar = 50 nm)

DISCUSSION

The therapeutic approach based on antibiotics has been threatened by the emergence of ESBL-producing *E. coli* worldwide in recent years because of their multidrug resistance properties. This problem has urged scientists to look for alternative therapeutic approaches to fight against ESBL-producing *E. coli* infectious diseases. One of the promising approaches is bacteriophage therapy that is the therapeutic and prophylactic use of lytic bacteriophages to treat pathogenic bacterial infections [12, 13, 14]. Here, we screened for a bacteriophage capable of inactivating ESBL-producing *E. coli*. Although several bacteriophages specific to ESBL-producing *E. coli* have been isolated [15, 16, 17], our work is still important because the previously isolated bacteriophages may not have infectivity against our ESBL-producing *E. coli* strain due to the high host specificity of the bacteriophages. Moreover, having many bacteriophages specific to ESBL-producing *E. coli* in hand can benefit the improvement of bacteriophage therapy against ESBL-producing *E. coli* by using them as cocktails of different bacteriophages that have broad spectrum against many strains of ESBL-producing *E. coli*.

In this study, ϕ UBU-ESBL, a bacteriophage specific to patient-derived ESBL-producing *E. coli*, was isolated from a wastewater sample collected from a hospital's wastewater treatment pond. The finding of ϕ UBU-ESBL in different place from which its specific bacterial host was isolated is not an unusual result because it has been known for a long time that bacteriophages are wide spread in the environment. Several previous works reported the isolation of bacteriophages from different places where their specific hosts exit. For examples, bacteriophage ϕ kpdr1 specific to *Klebsiella pneumoniae* DR1 derived from a hospitalized patient was isolated from a sewage water sample [6] and bacteriophage ST1 specific to *Salmonella* Typhimurium ATCC13311 derived from feces of a patient suffering from food poisoning was isolated from a water sample collected from a swine lagoon [11].

Bacteriophage host range is one of the parameters needed to be considered when a bacteriophage is selected to be used as a therapeutic agent. In this study, it was found that ϕ UBU-ESBL had a very narrow host range. It inhibited only its specific host, ESBL-producing *E. coli*, but not the other tested bacteria. The high host specificity of ϕ UBU-ESBL makes the bacteriophage a potential candidate for use as a therapeutic agent against ESBL-producing *E. coli*. Theoretically, it is harmless to the eukaryotic hosts undergoing bacteriophage therapy, and it should not affect the beneficial normal flora of the host. It also has few, if any, side effects, as opposed to drugs, and does not stress the liver [4].

In order to classify ϕ UBU-ESBL, information on its genome and morphology are required. The digestion of ϕ UBU-ESBL genome by the restriction enzyme *Bam*HI suggested that it was double stranded DNA. This result was confirmed by the nondigestibility of the genome by RNase A (an RNA digesting enzyme) and nuclease S1 (a single stranded DNA/RNA digesting enzyme). As examined by transmission electron microscopy, ϕ UBU-ESBL had an isometric head with a noncontractile tail. According to the International Committee on Taxonomy of Viruses, tailed bacteriophages with double stranded DNA are classified in the *Caudovirales* order [18]. This order contains three families including *Myoviridae* (with long, contractile tail), *Siphoviridae* (with long, noncontractile tail), and *Podoviridae* (with short tail). Based on its genomic and morphological characteristics, ϕ UBU-ESBL was tentatively classified as a member of the *Siphoviridae* family.

CONCLUSION

In conclusion, ϕ UBU-ESBL can be a potential candidate for being use as a therapeutic agent against ESBL-producing *E. coli* based on its high host specificity. This property benefits the bacteriophage to cause few, if any, effects on eukaryotic hosts and normal flora residing in the hosts. Furthermore, ϕ UBU-ESBL and other previously isolated ESBL-producing *E. coli* bacteriophages can be used together as bacteriophage cocktails to broaden inhibitory spectrum against many strains of ESBL-producing *E. coli*. However, more bacteriophage characterization and in vivo testing for the infectivity of ϕ UBU-ESBL are required in order to use it as a therapeutic agent. Some of them are underway in our laboratory.

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