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Isolation and characterization of lytic bacteriophages from sewage water

(Pengasingan dan pencirian bakteriofaj daripada air kumbahan)

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Abstract

Bacteriophages are virus-like particle that infect bacteria and can be found in all reservoirs populated by bacterial hosts, such as soil, sewage, sea water or even in the intestine of animals. Phages are very specific to the bacteria, thus they are suitable to be used as 'bacteria-killers', and subsequently they can be applied as biological control agents for the plant pathogens. From this study, a total of eight bacteriophages have been successfully isolated by using three different strains of *Escherichia coli* (TG1, ER2738 and BL21) as a host. They were characterized based on the morphology of the plaque forming, nucleic acid analysis and protein profile. Current study also showed that these phages might be in the range of lytic phages because they are similar to T-series bacteriophages. However, there was no lysogenic phage found in these sewage samples.

Introduction

Bacteriophages (phages) are obligate intracellular parasites that multiply inside the bacteria by making use of some or all of the host biosynthetic machinery (Elizabeth and Alexander 2005). They were discovered by British pathologists in London in 1915 by transmissible agent of '*Micrococcus*' colonies (Michael et al. 2003). Two years later, French Canadian microbiologist accidentally discovered the lysis formation of *Shigella* culture in the broth, which caused by phage.

There are two types of bacteriophage host range, monovalent and polyvalent. Polyvalent phages are able to attack two or more bacteria species, whereas monovalent phages are specific to one type of bacteria species (Kalmansom and Bronfenbrenner 1942). Phages are highly host specific and they only attack a particular group of bacteria species. The host specificity is dependent on the evolution of recognition system of the viruses, based on 'lock-and-key' theory (Kutter and Sulakvelidze 2005). The receptors on the bacteria host are recognized by the protein on the phage (Nester et al. 2004).

The phages are commonly named in reference to their host. Thus, the phage which attack the bacterium *Staphylococcus* is called staphylophage and those attack *Escherichia coli* is called coliphage. Most of the coliphages are made of DNA-containing isomeric or prolate icohedral head, often with contractile tail attached which serve as an adsorption and injection device to introduce the viral genome into the bacteria host (Elizabeth and Alexander 2005). When the phage attacks the bacteria, each of the phage will multiply and release several hundred new viruses, and the bacteria in the area surrounding the phages are destroyed, leaving a clear area or plaque on the

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agar. Each plaque corresponds to a single infective virus in the initial suspension (Ted and Christine 2004)

Since those phages are specific against the bacteria host, they have great potential to be used as biological control agent against certain plant pathogens. Previous studies have shown that phages can control *Ralstonia solanacearum* in tobacco (Tanaka et al. 1999) and, soft rot and fire blight associated with *Erwinia* spp. (Eayre et al. 1990). This study was conducted to isolate and characterize the potential phages from sewage water which can potentially be used as biocontrol agent against plant pathogens.

Materials and methods

Isolation and purification of phages

Sewage samples were collected from a sewage treatment plant at Puchong (Indah Water Konsortium Sdn. Bhd.). Three different types of *E. coli* strains [TG1 (*supE, hsd*Δ*5, thi*Δ*(lac-pro AB), F'[traD36, proAB*$^{+}$ *lacI*^{q}*, LacZ,* Δ*M15*]), ER2738 (F$'$ *pro A*$^{+}$*B*$^{+}$ *lac*$_{1}^{q}$ Δ (*lacz*) *m15 zzf*::*Tn*10 (*Tet*^{R})*/fhu Az gln* vΔ (*lac-proAB*) *thi*-1 Δ (*hsds-mcrB*) 5) and BL21 (*F- ompThsdSB (rB-mB-)galdcm*)] were used as a host for the amplification of the phages. Each of the *E. coli* culture (5 ml) was added into the fresh Luria Bertani (LB) broth (100 ml) containing Tetracyclin (5 mg/ml, Sigma). The mixture was incubated with shaking until the OD_{600} about 0.5.

Sewage sample (50 ml) was added into *E. coli* culture prepared previously and incubated overnight on the shaker at 37 °C, 250 rpm. The phage mixture was centrifuged at 10,000 rpm for 5 min and the supernatant was transferred to the new conical flask. The supernatant was filtered with membrane filter (0.45 µm, Whatman) to remove the unnecessary particles. The phage particles in the supernatant were precipitated by adding polyethylene glycol [6x PEG/NaCl (20% PEG 8000) and NaCl (2.5 M)]. The suspension was kept at 4 °C for 1 h and centrifuged at 13,000 rpm for 30 min at 4 °C. Finally, phage pellet was resuspended in TBS (50 mM Tris; 150 mM NaCl, pH 7.5).

Phage titration

A single colony of each *E. coli* (TG1, ER2738 and BL21) was inoculated into 5 ml of Luria Bertani (LB) broth and incubated with shaking until the OD_{600} about 0.5. Top agarose (1% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% $MgCl_2.6H_2O$, 0.7% agarose) was aliquoted (3 ml) into tubes and equilibrated at 45 °C in a water bath. Phage (10 µl) and mid-log phase *E. coli* cells were added to the equilibrated top agarose, mixed and poured onto a LB agar plate. The plates were allowed to cool and incubated overnight at 37 °C. Plaques formed were counted and the amount of phage was determined as plaque forming unit (pfu). All assays were performed in triplicates.

Extraction of single-stranded DNA

Phenol (50 µl, Sigma) was added to the phage suspension (100 µl) and the mixture was allowed to stand for 1 min at room temperature (~27 °C). The sample was centrifuged at 13,000 rpm for 5 min and the upper aqueous phase was transferred to the new tube. Chloroform (50 µl, Sigma) was added and mixed well by vortexing. The tube was allowed to stand for 1 min, centrifuged as above. The top phase was transferred to a new tube and Sodium acetate (300 µl, AmResco) was added. The mixture was incubated at room temperature for 30 min and then centrifuged at 13,000 rpm for 5 min at 4 °C. The supernatant was discarded and the DNA pellet was washed by adding ethanol [70% (v/v), 200 µl], vortexed briefly and centrifuged. The residual ethanol was allowed to evaporate by air-drying, and the DNA was dissolved in sterile distilled water (20 µl).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence

of a reducing agent (β-mercaptoethanol) is a technique used for the separation of polypeptide subunits according to their molecular weight. This agent is essential in order to break down the peptide bond of the phage protein. The separation in the gel generates bands representing the phages structural proteins. The distance of migration through the gel is directly related to the size of the protein. The electrostatic repulsion that is created by the binding of SDS buffer causes the protein to unfold into rod-like shape and generates the bands according to the different shape of protein. In this study, purified bacteriophages were mixed with 2x sample buffer (62.5 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol) and boiled for 10 min. The protein profile of phages was analysed on 15% polyacrylamide gels and stained with Coomassie brilliant blue (CBB) R-250 as described by Laemmli (1970).

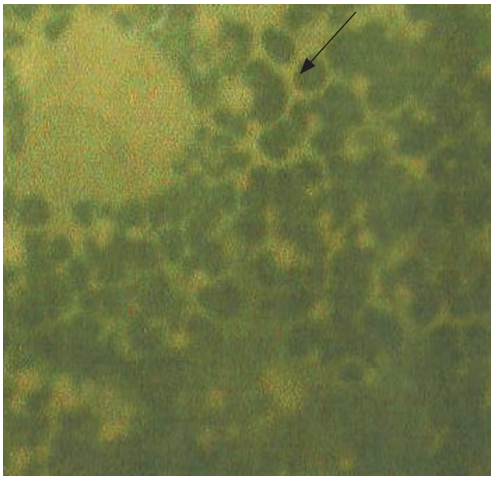

Plate 1. LB agar plate contains the plaque (with the arrow) showing the area infected by the phages

Results and discussion

The plaque caused by the phages varies in size and is characterized by the circular zone (*Plate 1*). The number of plaque forming unit from phage isolates were found to be as high as 10^{10} to 10^{12} pfu/ml (*Table 1*). The high PFU observed in this study could be due to the mid-log phase of *E. coli* used in the study which provided fresh growing cells resulting in enhanced production of progenies by the phages.

The single plaque is expected to vary in size and morphology because the *E. coli* cells had been infected by different type of phages. There are two common types of phages namely lytic (T-series) and lysogenic phages. The lytic phages normally cause lysis or clear condition to the host cells. On the other hand, the lysogenic phages formed the turbid condition which lysed the cells (Singleton 1992).

Lytic phages multiplied within host cell and lyse them (Singleton 1992). They undergo the lytic infection cycle where they proliferated in the host cells, and emerge from host cells by lysing process. During the process, phages will attach to the bacteria cell wall and inject their DNA into the cell. Phage DNA will replicate and genes encoding protein coat will be transcribed orderly using the host machinery system. At the end of the cycle, many viruses will

Table 1. Plaque morphology of isolated phages from sewage water

Host (E. coli strains)	Plaque morphology			
	Number (pfu/ml)	Turbidity	Diameter (cm)	Holo size (cm)
BL21	2.3×10^{11}	Clear	0.2 ± 0.2	–
BL21	1.2×10^{11}	Clear	0.1 ± 0.2	–
BL21	1.6×10^{11}	Clear	0.2 ± 0.2	–
BL21	1.3×10^{11}	Clear	0.4 ± 0.1	–
ER3738	1.4×10^{12}	Clear	1.0 ± 0.1	–
ER2738	2.1×10^{12}	Clear	1.0 ± 0.2	–
TG1	1.0×10^{10}	Clear	2.0 ± 0.5	–
TG1	1.05×10^{10}	Clear	4.0 ± 0.5	–

be produced and the *E. coli* will burst (Prescott et al. 2005). On the other hand, lysogenic phages integrate their chromosome into the chromosomal DNA of the host or establishing themselves as a plasmid, and enter the host cells in a harmless condition (Prescott et al. 2005).

The result of DNA extraction analysis of phages showed that they were more than 10,000 bp. The morphology and protein analysis showed that the isolated phages from *E. coli* strains ER2738, TG1 and BL21 were from a homologous family. Previous study reported that most of the T-series phages contain the DNA size of more than 10,000 bp. One example is T7 phage which consists of genome size of approximately 40,000 bp (Karam 2005). Due to the longer length of the DNA, T-series phages do not integrate into the bacterial chromosome. However, typical lysogenic phages such as M13 only consist of 6.4 bp long DNA and it can easily incorporate into the host cell chromosomes in order to generate their progenies without lysing the cells. Thus, isolated phages from sewage were most likely to be in the range of lytic phages which were similar to T-series bacteriophages.

The eight single isolated protein profiles revealed similar protein profiles which contain one major structural protein with the molecular weight ranging from 46 to 49 kDa, which represent the capsid protein. The result indicates that they have the similar capsid morphotype. In research conducted by Leiman et al. (2003), the composition of T4 prohead and mature head consist of major protein profile ranging from 46 to 56 kDa. Therefore, isolated phages in this study might come from lytic phages which are similar to T-series bacteriophages.

Conclusion

Sewage contains abundant *Escherichia coli* which serves as susceptible and highly adaptable host for the growth of bacteriophages. Eight isolates of phages were successfully isolated in this study and characterized based on morphology of the plaque, nucleic acid analysis and phage protein composition profile. These isolated phages may be in the range of lytic phages based on the DNA analysis and protein profile study. However, more precise studies using transmission electron microscope (TEM) analysis, DNA sequencing and restriction mapping need to be carried out to further characterize, identify and distinguish the isolated phages to the species level.

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Abstrak

Bakteriofaj ialah sejenis virus yang menyerang bakteria dan boleh dijumpai di merata-rata tempat, yang terdapat bakteria sebagai perumah, seperti di dalam tanah, air kumbahan, laut atau dalam sistem pencernaan haiwan. Faj sangat khusus kepada bakteria, dan menjadikannya sesuai sebagai 'pembunuh bakteria' dan seterusnya boleh digunakan sebagai agen pengawalan biologi bagi penyakit tanaman. Daripada kajian ini, lapan jenis bakteriofaj telah berjaya diasingkan daripada air kumbahan dengan menggunakan *Escherichia coli* (TG1, ER2738 dan BL21) sebagai perumah, dan dicirikan berdasarkan morfologi pembentukan plak, analisis asid nucleik dan profil protein. Kajian menunjukkan faj tersebut dikategorikan dalam kumpulan faj lisis kerana mempunyai ciri-ciri yang sama dengan T-series bakteriofaj. Namun, tiada faj dari kumpulan lisogen dijumpai dalam sampel kumbahan ini.

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