Isolation and Characterization of a Lysogenic phage from *Burkholderia pseudomallei*

การแยกและลักษณะเฉพาะของ ไลโซเจนิกฝาจจากเชื้อ *Burkholderia pseudomallei*

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ABSTRACT

Burkholderia pseudomallei is the causative agent of melioidosis, an infectious disease endemic in northeast Thailand. Lysogenic phages are viruses that integrated their DNA into host bacterial chromosomes. In this study, lysogenic phages were isolated from *B. pseudomallei* clinical isolates by mitomycin induction. Eighty percents of *B. pseudomallei* tested isolates produced phages. One of them, designated ΦP27 was further characterized by electron microscopy, host range analysis, growth characteristic and genome restriction analysis. Transmission electron micrograph of ΦP27 revealed that it possesses an isometric head of 73.33 nm in diameter with a non-contractile tail of 175 nm in length, which belong to the B1 morphotype of Siphoviridae family. This phage was able to lyse 51.1% of *B. pseudomallei* and 10% of *B. thailandensis* but not to *B. mallei*, *E. coli*, *K. pneumoniae* and *P. aeruginosa*. The nucleic acid of ΦP27 was double stranded DNA with approximately 42.89 Kb. This phage demonstrated a short latent period (20 min) and small burst size (16 particles) resulting to observed small plaques of phage on the host.

บทคัดย่อ

Burkholderia pseudomallei เป็นสาเหตุ ของโรคเมลิออยโดสิสซึ่งเป็นโรคติดเชื้อที่มีการระบาดในภาคตะวันออกเฉียงเหนือของประเทศไทย ไลโซเจนิกฝาจเป็นไวรัสที่แทรกดีเอ็นเอของมันเข้าไปในโครโมโซมของแบคทีเรียที่เป็นโฮสท์ การศึกษาครั้งนี้ได้ทำการแยกไลโซเจนิกฝาจจากเชื้อ *B. pseudomallei* ที่แยกได้จากผู้ป่วยโดยการกระตุ้นด้วย mitomycin C พบว่าเชื้อ *B. pseudomallei* ร้อยละ 80 สามารถปลดปล่อยฝาจ ฝาจหนึ่งสายพันธุ์ คือ ΦP27 ถูกนำมาศึกษาลักษณะเฉพาะโดยใช้กล้องจุลทรรศน์อิเล็กตรอน การวิเคราะห์โฮสท์ ลักษณะการเจริญเติบโต และการวิเคราะห์จีโนม ภาพจากกล้องจุลทรรศน์อิเล็กตรอนพบว่า ฝาจ ΦP27 มีลักษณะแคปซิดเป็นแบบสมมาตรเส้นผ่าศูนย์กลาง 73.33 นาโนเมตร และมีหางยาว 175 นาโนเมตร ซึ่งจัดอยู่ในกลุ่ม B1 ของสกุล Siphoviridae ฝาจ ΦP27 สามารถทำให้ *B. pseudomallei* แตกได้ ร้อยละ 51.1 และทำให้ *B. thailandensis* แตกได้

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ร้อยละ 10 แต่ไม่ทำให้ *B. mallei*, *E. coli*, *K. pneumoniae* และ *P. aeruginosa* แตก กรดนิวคลิอิกของ ΦP27 เป็นดีเอ็นเอสายคู่ ขนาดประมาณ 42.89 กิโลเบส และมีช่วงเวลาที่อยู่ในแบคทีเรียสั้นคือ 20 นาที มีปริมาณอนุภาคของฝาจหลังจากทำให้แบคทีเรียแตกคือ 16 อนุภาค ซึ่งมีผลทำให้สังเกตเห็น plaque ขนาดเล็กบนโฮสต์แบคทีเรีย

Key Words : *Burkholderia pseudomallei*, Lysogenic phage, Characterization
คำสำคัญ : *Burkholderia pseudomallei* ไลโซเจนิกฝาจ ลักษณะเฉพาะ

Introduction

Burkholderia pseudomallei is a gram-negative soil saprophyte and the causative agent of melioidosis, a disease that is endemic in northern Australia and southeast Asia particularly in northeast Thailand (White, 2003). Horizontal gene transfer of this pathogen contributes to the genetic diversity and may be an important determinant of virulence potential (Holden, *et al.*, 2004). Phage is a factor of horizontal gene transfer. It can convert its bacterial host from a nonpathogenic strain to a virulence strain or to a strain with increased virulence (Brussow and Hardt, 2004). The role of a lysogenic phage in virulence can be quite direct, in that some lysogenic phages carry toxin genes required for the host bacterium to cause disease (Wagner and Waldor, 2002). Previous studies (DeShazer, 2004; Ronning, *et al.*, 2010) have found that many *Burkholderia* species carried prophages. In this study, we have isolated and characterized a lysogenic phage from *B. pseudomallei* clinical isolates.

Materials and Methods

Bacterial strains and growth conditions

Fifty strains of *B. pseudomallei* used for isolation of lysogenic phages were obtained from Melioidosis Research Center. *B. thailandensis* (10), *B. mallei* (6), *E. coli* (6), *K. pneumonia* (6) and *P. aeruginosa* (6) isolates were used to examine the host range of a phage. *B. pseudomallei* P37 was used as a host for plaque assay and propagation of phages.

Nutrient broth (Oxoid Nutrient Broth No.2, 2%w/v) was used to grow the bacterial strains. Soft overlay agar for phage experiments comprises nutrient broth with 0.5% w/v agar (Oxoid agar No.1). For phage experiment nutrient broth and nutrient agar was supplemented with 400 µl/ml $CaCl_2$ (NB/$CaCl_2$, NA/$CaCl_2$). A mid log phase of bacterial propagating strain was prepared by inoculating a single colony into 3 ml NB and incubating with shaking at 200 rpm at 37˚C for 18 h. One percentage of overnight broth culture was inoculated into 3 ml fresh NB and incubated at 37˚C for 4 h with shaking at 200 rpm.

Isolation of lysogenic phages

Lysogenic phages were isolated from 50 isolates of *B. pseudomallei* by mitomycin C induction (de Saxe and Notley, 1978). Mitomycin C was added to the mid log phase of *B. pseudomallei* to a final concentration of 8 µg/ml. The mixture was incubated for 30 min and centrifuged at 2,500 rpm, 4°C for 20 min. Cell pellet was washed twice with normal saline solution and the cells were re-suspended with 5 ml NB/$CaCl_2$. The incubation was continued at 37°C with shaking for 2 h. Then, the culture was

centrifuged at 4°C, 2,500 rpm for 20 min. Finally, the supernatant phage lysates were collected and filtrated through 0.22 μm filter membrane. The presences of phages, phage purification and selecting of propagating strain were examined by measuring lytic activity using the plaque assay method (Kutter and Sulakvelidze, 2004). *B. pseudomallei* strain producing confluent lysis and not liberating phages spontaneously was chosen as a propagating strain for each phage.

Electron microscopy

Morphology of phage particles were observed by transmission electron microscopy of negatively stained preparation. A drop of phage lysate was placed on a carbon coated grid, negatively stained with 2% uranyl acetate, and examined by transmission electron microscopy (JEM-2100) at an accelerating voltage of 200KV. Electron micrographs were taken at a magnification of 80,000X. The phage size was determined from the average of three independent measurements (Kutter and Sulakvelidze, 2004).

Host range determination

The host range of the phages was examined by a spot test (Kutter and Sulakvelidze, 2004). Twenty microliters of a phage lysate (10^8 PFU/ml) was dropped onto the bacterial lawns prepared on NA/$CaCl_2$ plates. The plaque formation was observed following overnight incubation at 37ºC.

Genomic phage analysis

One hundred milliliters of phage suspension (10^8 pfu/ml) was precipitated with 10% polyethylene glycol (PEG, MW 8000), and centrifuged at 10,000 g, 4°C for 30 min. Phage pellet was re-suspended with 500 μl of SM buffer (10mM MgSO4, 100mM NaCl, 0.01% gelatin and 50mMTris-HCl) and treated with DNaseI (5U) at 37°C for 45 min to digest bacterial nucleic acid. Then, 5 μl of 10% SDS and 10 μl of 0.25 mM EDTA were added into phage suspension, incubated at 65°C for 15 minutes. Phage nucleic acid was extracted with the phenol:chloroform method and dissolved in TE buffer (Sambrook and Russell, 2001). Nucleic acid type was identified by agarose gel electrophoresis after treated with DNaseI and RNaseA. One micrograms of phage genome was digested at 37°C, overnight with 1U of *Hind*III and *Eco*RV restriction endonuclease enzymes. The resulting genome fragments were separated by electrophoresis on a 0.7% agarose gel using 1xTBE buffer (Sambrook and Russell, 2001). The gel was observed with ethidium bromide staining under UV light and photographed. The genome size was estimated based on the sum of molecular weight of fragments generated by digestion (Doi, *et al.*, 2003).

One step growth curve assay

The one-step growth experiments were modified from the protocol of Putzrath (Putzrath and Maniloff, 1977). A mid log phase culture (10 ml) of *B. pseudomallei* P37 was harvested by centrifugation and re-suspended in 2.5 ml of fresh NB (10^9 CFU/ml). Phage was added at a MOI of 0.005 and allowed to adsorb for 5 min at room temperature. The mixture was then centrifuged, pelleted cells were re-suspended in 10 ml of NB, and incubation was continued at 37°C. Samples were taken at 10-min intervals. The first set of samples

was immediately diluted and plated for phage titration. A second set of samples was treated with 1% (v/v) chloroform to release intracellular phages in order to determine the eclipse period.

Results

Isolation of lysogenic phages

Fifty isolates of *B. pseudomallei* were cross matching with phage lysates induced from each isolate. Lysogenic phages were detected in supernatants from 80% of *B. pseudomallei* isolates subjected to mitomycin C induction. A phage exhibiting strong lytic activity with clear plaques and could be propagated to a high titer was selected for characterization and designated as ΦP27. In order to increase the titer of a phage, the propagating host was necessary. The propagating host is the sensitive isolate producing confluent lysis and not liberating phages spontaneously. Thus, *B. pseudomallei* P37 wasused as a phage propagation host. ΦP27 produced clear plaques approximately 0.7 mm in size on *B. pseudomallei* P37 (Figure 1A).

Phage morphology

The electron micrographs showed the morphology of ΦP27 which belonged to the B1 morphotype of Siphoviridae family of Ackermann classification (Ackermann, 2003). It had icosahedral head approximately 73.33±0.05 nm in diameter attached to non-contractile tail approximately 175±0.057 nm in length and absence of collar and tail fibers (Figure 1B).

Host specificity of phage

Phage formed plaques on the lawn of its host. Phage ΦP27 could lyse 51.1% of *B. pseudomallei* and 10% of *B. thailandensis* but not lyse *B. mallei*, *E. coli*, *K. pneumoniae* and *P. aeruginosa*. It indicated that, ΦP27 was relatively broad host range in only *B. pseudomallei* and *B. thailandensis* isolates but not in other tested gram negative bacteria.

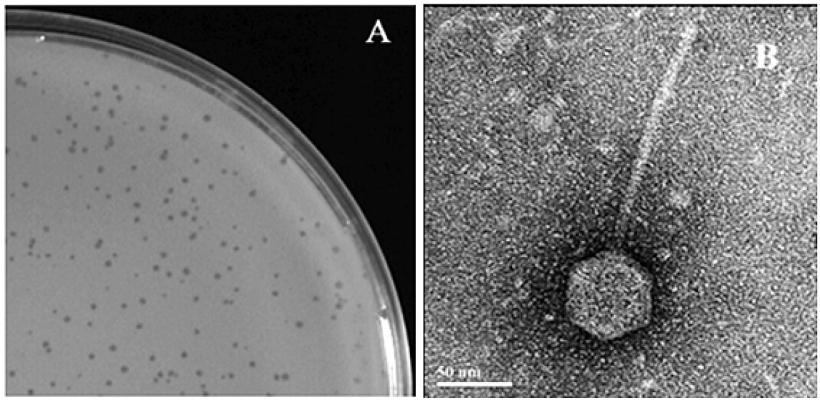

Figure 1 (A) Clear plaques of phage on the lawn of *B. pseudomallei* P37 by plaque assay method (B) Transmission electron micrograph of ΦP27 induced from *B. pseudomallei* negatively stained with 2% uranyl acetate. The picture was taken at an 80,000X magnification

Genomic phage analysis

Type of phage genome was analyzed by digestion phage nucleic acid with DNaseI or RNaseA enzymes. ΦP27 nucleic acids were digested by DNaseI but not RNaseA (Figure 2A). It indicated that ΦP27 genome was dsDNA. Based on the digestion profiles of *Hind*III and *Eco*RV restriction enzymes (Figure 2B), the genome size of ΦP27 was determined to be approximately 42.89 Kb.

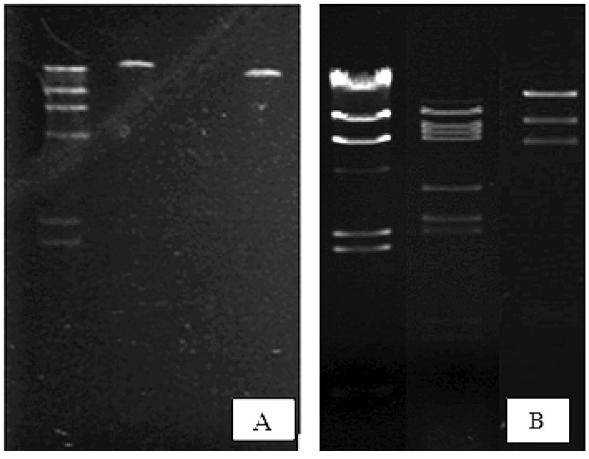

Figure 2 Agarose gel (0.7%) electrophoresis of phage DNA. (A): Nucleic acid type identification, lane 1; λ DNA/*Hind*III molecular weight marker, lane 2; ΦP27 DNA, lane 3; ΦP27 DNA treated with DNaseI, lane 4; ΦP27 DNA treated with RNaseA. B: DNA restriction pattern of ΦP27, lane 1; λ DNA/*Hind*III marker, lane 2-3; Phage DNA was digested with *Eco*RV and *Hind*III respectively.

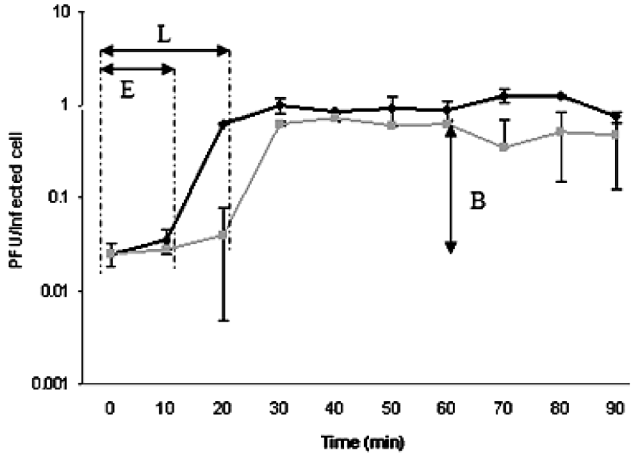

Figure 3 One step growths curve of ΦP27 on *B. pseudomallei* P37 at 37°C. The graph is shown the plaque forming unit per infected cell in untreated cultures (—■—) and in chloroform-treated cultures (—●—) of phage. The parameter of phage growth are indicated in the figure; E, eclipse period; L, latent period; B, burst size of phages.

One step growth curve analysis

The one step growth curve of ΦP27 on *B. pseudomallei* P37 was determined (Figure 3). The eclipse period, defined as the period between phage attachment and the assembly of the first progeny phage inside the bacteria was 10 minutes. The latent period, defined as the time interval between the time of adsorption and the beginning of the first burst was 20 minutes. The burst size of phage was approximately 16 particles per an infective phage. From this result, short latent period is related with small burst size of phage.

Discussion

In this study, the lysogenic phages could be isolated from 80% of *B. pseudomallei* clinical isolated by mitomycin C induction. The high number of lysogenic strains was in accordance with the previous report that 92% *B. pseudomallei* isolates were lysogeny (Manzeniuk *et al.*, 1994). The high prevalence of lysogeny might mediate a high potential for genetic transfer by transduction and contribute to the genetic diversity of *B. pseudomallei* (Ronning, *et al.*, 2010). The isolated phage (ΦP27) produced small clear plaques on a bacterial propagating strain. The rapid multiplication reflected in a short latent period and small burst size. However, these characters are depending on the relation of phage and specific host. When the propagation of phage population is active, the eclipse period should be short. The bacterial growth which displaying stationary phase or slow growth, it may be delay to the eclipse period. Similarly, longer latent periods are more optimal for phage population growth and also support larger phage burst (Abedon and Herschler, 2001; Abedon and Hyman, 2003). The purpose of classification is to summarize and categorize information and identification of a phage. Electron microscopic analysis of the lysates revealed that ΦP27 was classified in B1 morphotype of Shiphoviridae family. This result was similar to the previous study that described *B. pseudomallei* Φ1026b as Siphoviridae. However, most *Burkholderia* phages including *B. pseudomallei* Φ52237, ΦE12-2, Φ644-1 and Φ644-2 were classified in Myoviridae family (Ronning, *et al.*, 2010). Moreover, ΦC32 was classified in Podoviridae which considered to be a new morphotype of *B. pseudomallei* phage (Sariya *et al.*, 2006). Previously, *B. pseudomallei* phages were revealed that they had double stranded DNA and approximately 30-72 Kb in size with a low %G+C content (Ronning, *et al.*, 2010). In this study, we found that ΦP27 DNA fragments showed approximately 45 Kb after digestion by *EcoR*I and *Hind*III restriction enzymes. ΦP27 showed narrow host range which not specific to other tested gram negative bacteria. These may be depending on phage adhesin and bacterial receptor (Woods, 2002).

Conclusion

The lysogenic phages could be isolated from *B. pseudomallei* clinical isolates and ΦP27 was characterized. The knowledge related to the characterization of *B. pseudomallei* phage is important for phage-host interaction and require to further study including the roles of a lysogenic phage or a phage causing virulence and evolution of bacteria.

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