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ผลของแบคเทอริโอเฟจ 365A ในการยับยั้งเชื้อ *Burkholderia pseudomallei* ที่ดื้อต่อยาเซฟตาซิดิมในสภาวะแพลงโทนิคและสร้างไบโอฟิล์ม

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Effect of Bacteriophage 365A Against Ceftazidime Resistant *Burkholderia pseudomallei* in Planktonic and Biofilm conditions

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หลักการและวัตถุประสงค์: *Burkholderia pseudomallei* เป็นแบคทีเรียแกรมลบรูปแท่ง ที่เป็นสาเหตุของโรคเมลิออยโดสิส พบระบาดมากทางตอนเหนือของประเทศออสเตรเลียและภาคตะวันออกเฉียงเหนือของไทย เชื้อดื้อต่อยาปฏิชีวนะหลายชนิดรวมถึงยาเซฟตาซิดิม ซึ่งเป็นยาที่ใช้ในการรักษาโรค วัตถุประสงค์ของการวิจัยนี้เพื่อศึกษาลักษณะและความสามารถของแบคเทอริโอเฟจที่แยกได้จากเชื้อ *B. pseudomallei* ในการทำลายเชื้อและไบโอฟิลม์ของเชื้อ *B. pseudomallei* ที่ดื้อยาเซฟตาซิดิม

วิธีการศึกษา: คัดแยกและศึกษาโครงสร้างของแบคเทอริโอเฟจภายใต้กล้องจุลทรรศน์แบบส่องผ่าน ศึกษาชนิดสารพันธุกรรม ทดสอบความสามารถในการติดเชื้อแบคทีเรีย ด้วยวิธี spot test ทดสอบความสามารถของแบคเทอริโอเฟจในการยับยั้งเชื้อ *B. pseudomallei* ที่ดื้อต่อยาเซฟตาซิดิม ด้วยวิธี time kill assay รวมถึงการยับยั้งการสร้างไบโอฟิล์มของเชื้อ *B. pseudomallei* ที่ดื้อต่อยาเซฟตาซิดิม ด้วยวิธีการ colorimetric method

ผลการศึกษา: โครงสร้างของแบคเทอริโอเฟจ 365A ประกอบด้วยหัวรูปทรงหลายเหลี่ยมและหางที่ยืดหดได้ และมีดีเอ็นเอเป็นสารพันธุกรรม จึงจัดอยู่ใน family *Myoviridae* ความสามารถในการเข้าทำลายเชื้อ *B. pseudomallei* ที่ดื้อต่อยาเซฟตาซิดิมได้ร้อยละ 100 เปอร์เซ็นต์ (5 สายพันธุ์) นอกจากนี้ยังสามารถลดปริมาณเชื้อ *B. pseudomallei* ที่ดื้อยาเซฟตาซิดิม ในสภาวะแพลงโทนิกได้ 2 log units และ

Background and Objective: *Burkholderia pseudomallei* is a Gram negative bacilli bacteria that causes melioidosis. The endemic areas are in Northern Australia and Northeastern Thailand. Currently, bacteria resistance to many antibiotics, including ceftazidime, the drug of choice to treat melioidosis. This study investigated the characteristics and capabilities of bacteriophage isolated from *B. pseudomallei* to kill and reduce biofilm formation in ceftazidime resistant *B. pseudomallei.*

Methods: The bacteriophage was spontaneously isolated from *B. pseudomallei* 365A. It was characterized the structure by transmission electron microscope and identified nucleic acid type. Host range determination of bacteriophage was performed by spot test. Capability of bacteriophage to kill and reduce biofilm formation in ceftazidime resistant *B. pseudomallei* was performed by time kill assay and colorimetric method, respectively

Results: Bacteriophage 365A structure composed of icosahedral head and contractile tail with tail fibers. It had DNA as a genetic material, thus it belongs to *Myoviridae* family. Bacteriophage 365A was able to lysed 100% of ceftazidime resistant *B. pseudomallei* tested isolates. Bacteriophage 365A also reduced 2 log units of ceftazidime resistant *B. pseudomallei* in planktonic conditions and reduced 60-68% of biofilm formation.

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ลดการสร้างไบโอฟิลม์ของเชื้อ *B. pseudomallei* ที่ดื้อยาเซฟตาซิดิมได้ร้อยละ 60-68

สรุป: แบคเทอริโอเฟจ 365A ที่แยกได้จาก *B. pseudomallei* สายพันธุ์ 365A มีความสามารถในการลดปริมาณเชื้อ *B. pseudomallei* ที่ดื้อยาเซฟตาซิดิมในสภาวะแพลงโทนิกและลดการสร้างไบโอฟิลม์ได้ จึงอาจเป็นทางเลือกใหม่ที่จะนำแบคเทอริโอเฟจ 365A มาประยุกต์ใช้ในการควบคุมเชื้อ *B. pseudomallei* ที่ดื้อยาเซฟตาซิดิมในอนาคต

คำสำคัญ: *Burkholderia pseudomallei*, ดื้อยาเซฟตาซิดิม, แบคเทอริโอเฟจ, ไบโอฟิล์ม

Conclusion: Bacteriophage 365A isolated from *B. pseudomallei* strain 365A has potential to reduce ceftazidime resistant *B. pseudomallei* in planktonic conditions and can also reduce biofilm formation. According to efficiency of bacteriophages 365A, it may possibly use bacteriophage 365A to control ceftazidime resistant *B. pseudomallei*.

Keywords: *Burkholderia pseudomallei*, ceftazidime resistant, bacteriophage, biofilm

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Introduction

Burkholderia pseudomallei is a facultative intracellular Gram negative saprophytic bacterium that caused melioidosis which endemic areas are Northern Australia and Southeast Asia. This organism is present in the environment in a defined geographic distribution, and the infection is acquired through bacterial inoculation of wounds, inhalation, and ingestion. In Thailand, melioidosis is most frequently reported from the northeastern region where it is the most common cause of community-acquired bacteremia and mortality rate is approximately 40%[1]. It was classified as category B select agents of bioterrorism. *B. pseudomallei* exhibit intrinsic antibiotics resistance includes ampicillin, penicillin, first-generation and second-generation cephalosporin, gentamicin, streptomycin, tobramycin, polymyxin. Moreover, it also resistance to ceftazidime which is a drug of choice for melioidosis treatment[2]. This pathogen can be grown microcolonies and biofilm that act as a shield to protect itself from stress condition such as antibiotic, low nutrient, and immune system. Interestingly, biofilm was associated with antibiotic resistant, persistent infections and relapse in many cases of melioidosis patients[3].

Bacteriophages or phages are the viruses of bacteria and abundant in the nature. They used bacterial cell and material inside host cell for propagation. Bacteriophage can undergo two different life cycles: lytic cycle and lysogenic cycle. In lytic cycle, at the final step of replication bacteriophage can make bacterial cell lysis, release new bacteriophages progeny inside out and can kill bacteria. While lysogenic cycle, bacteriophage can integrate their own genome into bacterial chromosomes and enable the replication when bacteria have reproduction and persist in bacterial descendant. However, both life cycles can be switched depending on various factors such as exposed to chemical or UV light[4]. Interestingly, they infect only specific host and are capable of killing drug-resistant bacteria. Their safety for application in food, animals, and humans has been demonstrated[5]. From this ability bacteriophage was possible to use as an antibacterial[6]. Previously, six lytic bacteriophages that lysed *B. pseudomallei* including ST2, ST7, ST70, ST79, ST88, and ST96 were isolated from soil in Khon Kaen province. Bacteriophages ST79 reduced 4 log units of *B. pseudomallei* strain P37 in planktonic condition at 4 h after bacteriophages were added. In addition, lytic bacteriophage ST79 has high potential to reduce approximately 80% of biofilm formation in *B. pseudomallei* strain P37[7]. Several lysogenic bacteriophages have been isolated from *B. pseudomallei* includes Ø1026b, ØP27 and ØX216[8-10]. The bacteriophage ØP27 was isolated from *B. pseudomallei* by mitomycin C induction and showed broad host range in *B. pseudomallei* and *B. thailandensis* isolates. It lysed 51.1% of *B. pseudomallei* and 10% of *B. thailandensis* strain tested but not lysed *B. mallei* and other gram negative bacteria. Kvitko and colleagues have found a P2-like bacteriophage ØX216 which was spontaneously

form clear bacteriophage plaques from *B. pseudomallei* environmental isolate E0237 after plating of overnight liquid cultures on agar plates. Bacteriophage ØX216 show ability to infect 78% of all *B. pseudomallei* strains tested and also infects *B. mallei*, but not other *Burkholderia* species, including the closely related *B. thailandensis* and *B. oklahomensis*[10].

The ability of bacteriophage to kill bacteria with specifically and advantages over antibiotic is very interesting and possible to use for antibiotic resistant bacteria[11]. For instance, research from Jamal and colleagues reported about bacteriophage MJ1 that was isolated from sewage water showed good capability to reduce the numbers of *Escherichia coli* 3 that was multi-drug resistant strain in planktonic cells and bacteriophage BØ-R1215 and BØ-R2315 were strongly active against host bacteria carbapenem resistant *Acinetobacter baumannii in vitro*[12,13]. Therefore, bacteriophages may be the one of suitable alternative agent active against *B. pseudomallei* in both planktonic and biofilm condition. In this study new bacteriophage of *B. pseudomallei* was isolated from *B. pseudomallei* strain 365A and characteristic of bacteriophage was determined. The ability of lytic bacteriophages to kill ceftazidime resistant *B. pseudomallei* and biofilm reduction ability of lytic bacteriophage was investigated. The efficacy of bacteriophage may be consider as an alternative treatment or use as a bio-control tool applications in the future.

Materials and Methods

Bacterial strains and growth condition

B. pseudomallei 365A was used for bacteriophage isolation due to it is spontaneously forms clear plaques on its own bacterial lawn. *B. pseudomallei* 365A was intermediate resistance to ceftazidime with MIC (minimum inhibitory concentration) 16 µg/ml. *B. pseudomallei* P37 was used for bacteriophage propagation and detection[14]. *B. pseudomallei* 365A was grown to mid-log phase (approximately 10^8 CFU/ml) by added a 1% inoculum of an overnight culture into a nutrient broth and incubated in 37 ºC, 200 rpm for 4 h. Ceftazidime resistant *B. pseudomallei* including 316C, 979B, EPMN34, and, EPMN159 were characterized as ceftazidime resistant strains were selected to evaluate effect of bacteriophage to reduce numbers and biofilm formation[15]. Twenty-two strains of clinical *B. pseudomallei*, 5 isolates of *Burkholderia thailandensis*, 5 isolates of *Burkholderia mallei, Burkholderia cepacia* and pathogenic Gram-negative and Gram-positive bacteria including *Pseudomonas aeruginosa, Escherichia coli, Salmonella typhimurium, Klebsiella pneumoniae, Acinetobacter baumannii, Stenotrophomonas maltophilia, Staphylococcus aureus,* β-*Streptococcus* group B*, Enterococcus* spp*., Bacillus cereus, Listeria monocytogenes* and *Ralstonia solanocearum* were used for bacteriophage host range determination.

Bacteriophage isolation, detection and purification

Bacteriophage was isolated from ceftazidime resistant *B. pseudomallei* strains 365A. Briefly, single colony of *B. pseudomallei* strain 365A was inoculated into 3 ml of fresh nutrient broth and incubated at 37 °C 200 rpm for 24 h. Bacterial cells and cell debris were removed by centrifugation at 10,000 *xg* for 5 min. Supernatant was filtrated through 0.2 µm membrane filters and stored at 4 °C as bacteriophage lysate until used. The presence of bacteriophages was investigated by spot test[16]. Mid-log phase of *B. pseudomallei* P37 culture was adjusted to provide 0.5 McFarland and flushed on NA/$CaCl_2$ plate, the excess volume was removed and allowed it dry. Twenty microliters of bacteriophage lysate was dropped onto the plate and incubated at 37 °C for 16-18 h and finally clear zones formation was observed. Bacteriophage purification was performed by soft agar method[17]. Each identity isolated plaque was cored out by a sterile Pasteur pipette and placed into SM buffer, gently on rotator (Biosan, Latvia) for 1 h and centrifuged at 2500 *xg*, at 4 °C for 20 min. Supernatants was filtrated through 0.22 µm membranes filter and purified by soft agar method 2 times. The purification step was repeated three times to ensure the purity of the bacteriophage stock and purified bacteriophage lysate was stored at 4 °C until used.

Bacteriophage propagation and titration

Lysogenic bacteriophage was propagated in *B. pseudomallei* strain P37 by broth lysis culture methods[14].

Briefly, the overnight culture of *B. pseudomallei* strain P37 propagating strain was inoculated into 100 ml of fresh nutrient broth with 1 % inoculums and incubated at 37 °C for 4 h with 200 rpm shaking to let bacteria grow until mid-log phase. Purified bacteriophage suspension was added to give a MOI of 0.1 and $CaCl_2$ was added to give a final concentration of 400 µg/ml into mid-log phase culture flask. The liquid mixture was further incubated at 37 °C, 200 rpm for 5-6 h. The culture mixture was centrifuged at 4,000 *xg* at 4 °C for 40 min to remove the bacterial cells and cell debris. The supernatant was transferred to new conical tube and centrifuged again. Finally, the supernatant was filtrated through 0.2 µm membrane filter and bacteriophage titration was performed by spot test technique. In order to count number of propagated bacteriophage 365A, spot test was performed. Bacteriophage lysate was 10-fold serially diluted in SM buffer. A mid-log phase culture of *B. pseudomallei* P37 was adjusted to 0.5 McFarlane by 1× PBS buffer and lawn on NA/$CaCl_2$ agar. Twenty microliters of each serial dilution of bacteriophage lysates was spotted on NA/$CaCl_2$ agar and incubated at 37 °C for 18 h. Finally clear plaques were counted and bacteriophage titer was calculated to PFU/ml[14].

MIC determination

The MIC was performed in 96-well microtiter plates[18]. Ceftazidime was 2-fold serially diluted in Mueller Hinton broth (MHB) to concentrations 0.5-1024 µg/ml in 100 µl per well. A single colony of each ceftazidime resistant *B. pseudomallei* strains 316C, EPMN34, EPMN159, 979B, and 365A were grown on Ashdown's agar, inoculated into 3 ml of MHB and incubated at 37 °C, 200 rpm for 18 h. The culture was further diluted to provide final inoculums density of 2×10^5 CFU/ml in MHB, which was verified by the total viable count. The final inoculums (50 µl) were added in each well of 96-well microtiter plate. Wells containing only media and culture-free ceftazidime was included as negative controls. All samples were performed in triplicate. Plates were incubated at 37 °C for 24 h and the MIC was read according to the criteria established by the National Committee for Clinical Laboratory Standards (NCCLS)[19].

Host range determination

Host range of bacteriophage 365A was determined in Gram negative and Gram positive bacteria including ceftazidime resistant *Burkholderia pseudomallei, Burkholderia pseudomallei, Burkholderia thailandensis, Burkholderia mallei, Burkholderia cepacia, Ralstonia solanacearum, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhimurium, Klebsiella pneumonie, Acinetobacter baumannii, Stenotrophomonas maltophilia, Staphylococcus aureus,* β-*Streptococcus* group B*, Enterococcus* spp.*, Bacillus cereus* and *Listeria monocytogenes.* An overnight culture of each bacteria was inoculated into new 3 ml of nutrient broth with 1% inoculum and incubated at 37 °C, 200 rpm for 4 h. A mid-log phase of each bacteria was adjusted to 0.5 McFarland with 1× PBS and spread on the dry surface of NA/$CaCl_2$ agar. Twenty microliter of each bacteriophage suspension (approximately 10^8-10^9 PFU/ml) was spotted and incubated at 37 °C for 18–24 h. The results were recorded as negative if there was no plaque and positive when clear plaques were observed[14].

Bacteriophage morphology by transmission electron microscopy

To visualize bacteriophage 365A morphology by using transmission electron microscopy, the bacteriophage particles were stained with negative staining method[20]. A 10 µl of purified bacteriophage suspension (more than 10^8 PFU/ml) were placed on carbon coated formvar grid for 30 min. The excess bacteriophages suspension was removed by using filter paper and air dried. The negative stain was done by applied 10 µl of 1% uranyl acetate onto bacteriophage particles on the grid for 10 min and removed the excess stain before let it dried at room temperature. Bacteriophage morphology was observed under a transmission electron microscope (FEI, China) and pictures of bacteriophage 365A were taken.

Bacteriophage DNA isolation and restriction endonuclease analysis

Bacteriophage DNA isolation was performed with the modified protocol for lambda bacteriophage DNA extraction[21]. One hundred milliliters of bacteriophage

365A suspension (more than 10^8 PFU/ml) was added gradually with 30% polyethylene glycol (PEG) to final concentration 10% of PEG and gently mixed at 4 °C for overnight, bacteriophage suspension was centrifuged at 9,000 ×g, 4 °C for 30 min and the pellet was resuspended in 10 ml of SM buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgSO_4$ and 0.01% gelatin solution). Contaminated bacterial DNA was removed by DNase I. Bacteriophage nucleic acids was extracted with the phenol : chloroform method and dissolved in TE buffer. Finally, bacteriophage DNA was digested with *Xho*I, *Sac*I and *Kpn*I and patterns were observed by 0.7% agarose gel electrophoresis.

Effect of bacteriophage 365A on ceftazidime resistant *B. pseudomallei* growth

The *in vitro* efficacies of bacteriophage 365A to *B. pseudomallei* P37 (susceptible strain) and ceftazidime resistant strains *B. pseudomallei* including strains 316C, 979B (MIC 128 and 64 µg/ml) were determined by a time-kill assay[22]. In brief, a mid-log growth phase of *B. pseudomallei* strains P37, 316C and 979B were diluted to 10^7 CFU/ml in 3 ml of NB/$CaCl_2$. Bacteriophage 365A was added at MOI of 0.1, 1, and 10 and the samples were incubated at 37 °C 200 rpm. The numbers of bacteria remaining at 0, 2, 4, 6, 8, 12 and 24 h were investigated by a plate count technique. The assay was performed in three independent experiments.

Effect of bacteriophage on biofilm formation of ceftazidime reristant *B. pseudomallei*

The efficacy of the bacteriophage 365A on ceftazidime resistant *B. pseudomallei* biofilm formation was determined in a 96-well polystyrene flat-bottom plate using a colorimetric method[17]. Briefly, 100 µl of mid-log phase culture of ceftazidime resistant *B. pseudomallei* was grown in Luria-Bertani (LB) and inoculated into 2× LB. A 100 µl aliquot of inoculated medium (10^7 CFU) was added to each well, followed by 100 µl of bacteriophage 365A in SM buffer (50 mM Tris HCl pH 7.5, 0.1 M NaCl, 8 mM $MgSO_4$ and 0.01% (w/v) gelatin) at MOI of 0.01, 0.1, 1 and 10, and then the plate was incubated at 37 °C for 24 h. Supernate in each well was removed and replaced with 100 µl of bacteriophage at the same MOI and 100 µl of 2× LB. After incubation at 37 °C for an additional 24 h, plate was washed three times with 200 µl of sterilized water. The attached bacterial cells in each well were fixed with 200 µl of absolute methanol for 15 min, removed all of fluid and dried at room temperature. Each well was stained with 200 µl of 2% crystal violet for 15 min, rinsed with running tap water and dried at room temperature. The crystal violet in each well was dissolved in 200 µl of 33% (v/v) glacial acetic acid and optical density (OD) of each well was measured at 600 nm using the microtiter plate reader. Negative controls contained 2× LB with SM buffer and untreated controls contained bacterial culture and SM buffer were included. Each experiment was performed in three independent with twice repeats each. The percent biofilm reduction was calculated from the formula: [(OD_{595} nm of untreated control – OD_{595} nm of bacteriophage treatment)/OD_{595} nm of untreated control] x 100.

Results

Plaque morphology of *B. pseudomallei* bacteriophage 365A

A bacteriophage was isolated from *B. pseudomallei* 365A and designated as 365A. It produced clear plaque on plaque assay plate. Single plaque was purified by soft agar method. Bacteriophage produced clear plaques of about 1.5 mm in diameter on *B. pseudomallei* P37 lawn as shown in figure 1.

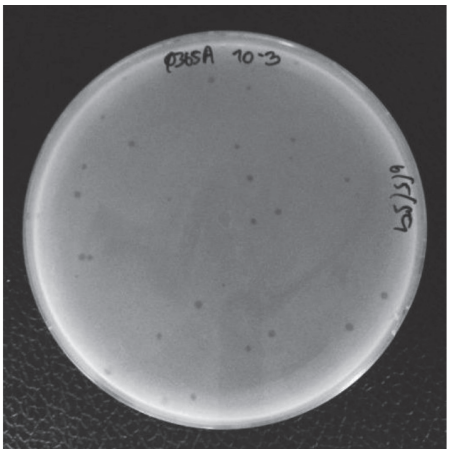

Figure 1 Plaque formation of bacteriophage 365A on lawn of *B. pseudomallei* P37

Host range determination

In order to determine the specificity of bacteriophage 365A to ceftazidime resistant *B. pseudomallei* and others, spot tested was performed. Based upon spot testing results, the bacteriophage 365A had lytic activity against 100% of ceftazidime resistant *B. pseudomallei* and 77% of ceftazidime sensitive *B. pseudomallei* strains tested. Bacteriophage 365A had a wide host range among clinical *B. pseudomallei* isolates. However, it could be lysed 40% and 60% of *B. pseudomallei* closely related species includes *B. thailandensis* and *B. mallei,* respectively. Interestingly, bacteriophage 365A cannot form plaque on other pathogenic Gram-negative or Gram positive bacteria that tested in this experiment (Table 1).

Table 1 Host range determination of bacteriophage 365A on *B. pseudomallei* and closely related species, other Gram positive, and Gram negative bacteria

Bacteria	Plaque formation
Ceftazidime resistant *Burkholderia pseudomallei* (5 strains)	100% (5/5)
Burkholderia pseudomallei (22 isolates)	77% (17/22)
Burkholderia thailandensis (5 isolates)	40% (2/5)
Burkholderia mallei (5 isolates)	60% (3/5)
Burkholderia cepacia	-
Ralstonia solanacearum	-
Pseudomonas aeruginosa	-
Escherichia coli	-
Salmonella typhimurium	-
Klebsiella pneumoniae	-
Acinetobacter baumannii	-
Stenotrophomonas maltophilia	-
Staphylococcus aureus	-
β-*Streptococcus* group B	-
Enterococcus spp.	-
Bacillus cereus	-
Listeria monocytogenes	-

-, No plaque

Nucleic acid type and restriction endonuclease analysis

Nucleic acid of bacteriophage 365A was digested with DNase I but it was not digested by RNase A, suggesting that its genetic material is double-stranded DNA. Furthermore, bacteriophage 365A DNA was digested with *Xho*I, *Sac*I and *Kpn*I, the restriction enzyme digestion pattern of bacteriophage 365A showed different pattern when digested with *Xho*I, *Sac*I and *Kpn*I as shown in Figure 2. The estimated genome was approximately 28 kb.

Morphology of bacteriophage particles

The bacteriophage 365A morphological characterization was done by using transmission electron microscopy. TEM observations of bacteriophage 365A revealed icosahedral head (50 nm in diameter) and contractile tail (148 nm in length and 18 nm in width) with tail fibers was shown in Figure 3. According to guidelines of the International Committee on Taxonomy of Viruses (ICTV), bacteriophage typing is based on morphology and nucleic acid types. Therefore, bacteriophage 365A belongs to the order *Caudovirales* and family *Myoviridae*[21].

Effect of bacteriophage 365A on ceftazidime reristant *B. pseudomallei* growth

Antibacterial activity of bacteriophage 365A against *B. pseudomallei* P37 in planktonic condition at different MOIs was similar. After 4 h of treatment with bacteriophage, viable counts of bacteria were rapidly decreased approximately 3 log units when compared with control. However, after 6 h the bacteria were regrowth, until 24 h the number of bacteria were equally in all tested condition as shown in Figure 4A.

In case of ceftazidime resistant *B. pseudomallei* strains 316C and 979B, the result showed bacterial number was not difference after added bacteriophage for 2 h when compared with control. However, after 4 h of treatment with bacteriophage, viable counts of bacteria were reduced approximately 2 log units as shown in Figures 4B and 4C, respectively. Similar to *B. pseudomallei* P37, bacteria were able to regrow after bacteriophages were added for 6 h and until 24 h.

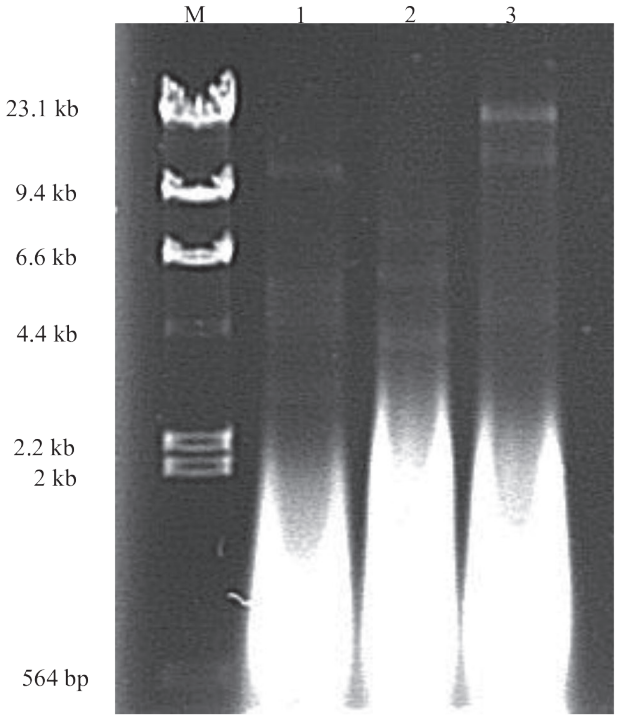

Figure 2 Restriction enzyme digestion pattern of bacteriophage 365A. Lane M; Marker (lambda bacteriophage DNA treated with *Hin*dIII), Lane1; Bacteriophage 365A nucleic acid was treated with *Xho*I, Lane 2; Bacteriophage 365A nucleic acid was treated with *Sac*I and Lane 3; Bacteriophage 365A nucleic acid was treated with *Kpn*I.

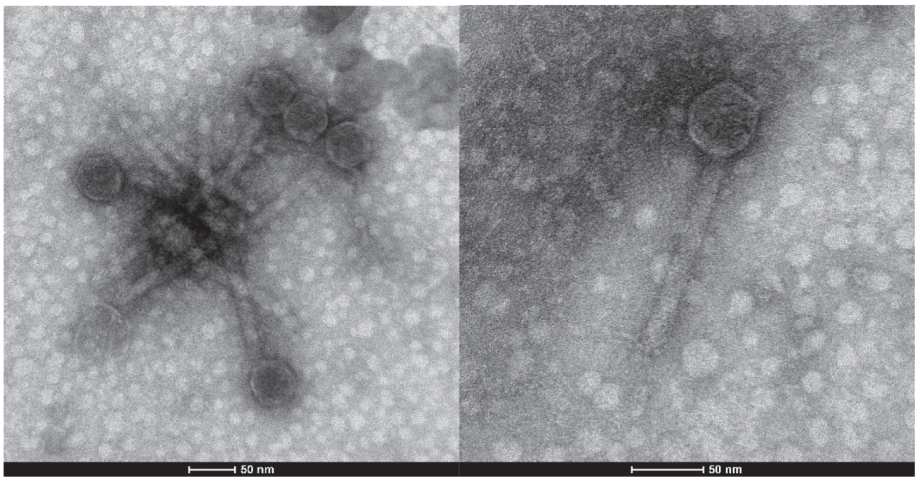

Figure 3 Transmission electron micrograph of bacteriophage 365A. The bar represents 50 nm in length. Bacteriophage was negatively stained with 2% uranyl acetate, it shown icosahedral head, contractile tail and tail fibers.

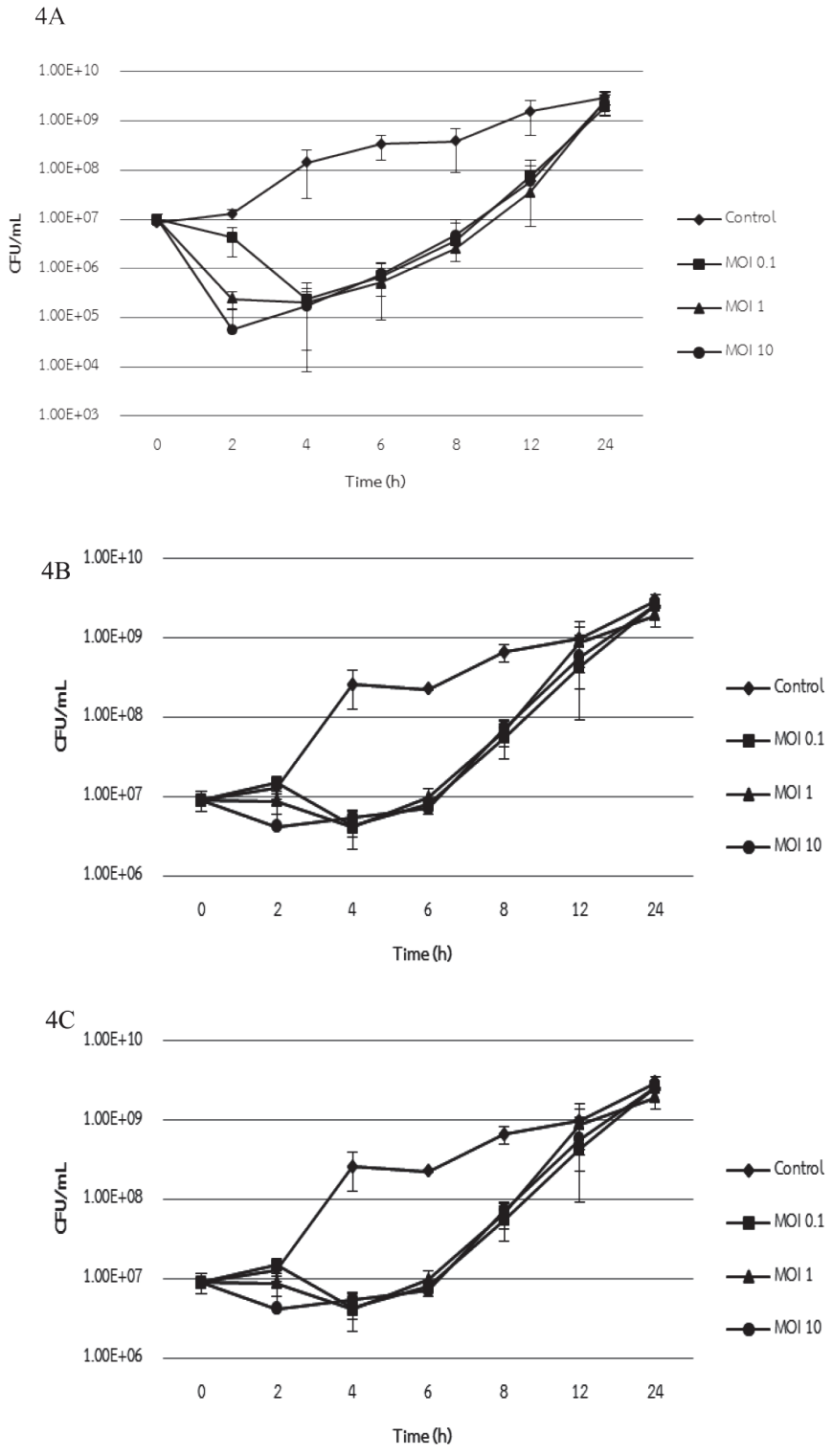

Figure 4 Bacterial growth inhibition of bacteriophage 365A against ceftazidime resistant *B. pseudomallei*. Bacteriophage 365A at MOI of 0.1, 1 and 10 were added to 10^7 CFU/ml of *B. pseudomallei* P37 (susceptible strain) (4A), *B. pseudomallei* 316C (4B), *B. pseudomallei* 979B (4C). Control was a bacterium without bacteriophage infection. Results are expressed as mean ± SD.

Effect of bacteriophage on biofilm formation of ceftazidime reristant *B. pseudomallei*

The biofilm reduction ability of bacteriophage 365A was investigated against 3 isolates of *B. pseudomallei* including P37 (susceptible isolate), 316C (MIC 128 µg/ml), 979B (MIC 64 µg/ml). The different concentrations of bacteriophages on biofilm reduction (MOI 0.1, 1.0, and 10) compared with the untreated controls were tested. The result showed that biofilm reduction when treated with bacteriophage 365A at MOI 10 could significantly reducel 80% of biofilm formation in *B. pseudomallei* P37, 68% and 60% in ceftazidime resistant *B. pseudomallei* strains 316C and 979B respectively, when compared to untreated group ($p < 0.05$) as shown in figure 5.

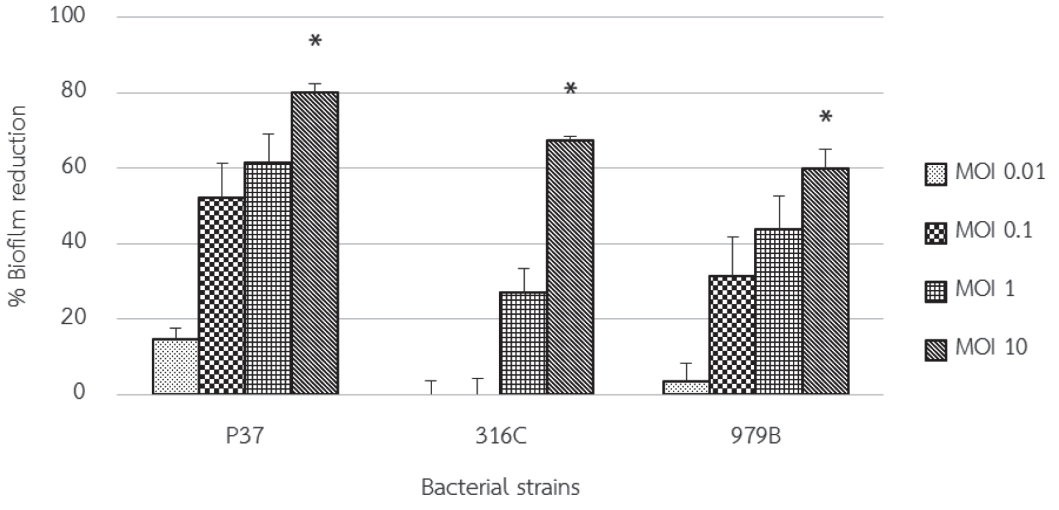

Figure 5. Effect of bacteriophage 365A on *B. pseudomallei* biofilm formation. Biofilm was measured by a crystal violet assay after challenge with bacteriophages at MOI of 0.01, 0.1, 1 and 10. Results are expressed as mean ± SD percent biofilm relative to untreated controls.

Discussions

In this study, bacteriophage 365A was spontaneously found when ceftazidime resistant *B. pseudomallei* 365A was used for bacteriophage screening by soft agar method. Clear plaques were observed on ceftazidime resistant *B. pseudomallei* 365A lawn. Since bacteriophage 365A was spontaneously produced from ceftazidime resistant *B. pseudomallei* strain 365A, thus it was lysogenic bacteriophage. However, it had ability to lyse *B. pseudomallei* and closely related species such as *B. mallei* and *B. thailandensis*. Bacteriophages may bactenal host either through the lytic or the lysogenic cycles. Whereas the lytic cycle leads to lysis of the bacterial cell, in the lysogenic cycle the bacteriophage genome integrates into the bacterial genome, and the lysogenized bacterium becomes immune to further infection by the same bacteriophage.

Transmission electron microscopic revealed that bacteriophage 365A structure was classified as *Myoviridae* family which similar to several report informed most *Burkholderia* bacteriophages was belong to *Myoviridae* family such as bacteriophages ST2, ST7, ST70, ST79, ST88 and ST96[14]. However, *Burkholderia* bacteriophages can belong to *Siphoviridae* family such as bacteriophage 1026b and bacteriophage P27 and also *Podoviridae* family such as bacteriophage C32[9,23].

Bacteriophage 365A has higher ability to reduce the numbers and biofilm of *B. pseudomallei* P37 which was a susceptible strain than 316C and 979B in planktonic and biofilm condition may be due to ceftazidime resistant strains *B. pseudomallei* might have some mechanism to resist bacteriophage such as the compatible of ligand of bacteriophage and receptor on bacterial cell surface.[24] The antimicrobial activities

of bacteriophages 365A at different MOIs against ceftazidime resistant *B. pseudomallei* shown high MOI reduced bacterial numbers more than lower MOI, suggesting that its lytic activity were dose dependent manner. However, when bacteriophages 365A was incubated for longer 12 and 24 h, bacterial numbers were increased similar to control condition. The phenomenon possibly caused by changed into lysogenic or other mechanisms. The other situation that may support bacterial regrowth is tube shaking, due to it can provide bacterial cells more expose to O_2 and promote bacterial growth. A cocktail of bacteriophages possibly prevented regrowth of bacteriophage-treated *B. pseudomallei,* but still needs more investigation about period of incubation and MOIs of cocktail bacteriophages.

Biofilm is a community of microbial cells that attached a wide variety of surfaces, including living tissues, in dwelling medical devices, soil environment and natural aquatic systems. The biofilm is a key part of pathogenic bacteria that facilitate the survival of pathogenic bacteria under various stress conditions such as desiccation, limited nutrient availability. *B. pseudomallei* also produced biofilm which related to antibiotic resistance. Bacteriophages were determined ability to reduce biofilm formation in ceftazidime resistant *B. pseudomallei*. In planktonic condition, after 6 h *B. pseudomallei* was able to regrowth, it may be due to bacteria possibly carrying lysogenic bacteriophage. Bacteriophage 365 had higher ability to reduce the numbers and biofilm of *B. pseudomallei* P37 which was a susceptible strain than 316C and 979B in both planktonic and biofilm condition may be due to ceftazidime resistant strains *B. pseudomallei* might have some mechanism to resist bacteriophage such as the compatible of ligand of bacteriophage and receptor on bacterial cell surface, adsorption blocking, intracellular restriction modification system or abortive infection[24]. The evaluated ability of bacteriophage to reduce biofilm formation by added bacteria and bacteriophage in the same time point may be should further investigate in bacteria that already formed biofilm. The observed reduction of *B. pseudomallei* biofilm in a dose dependent manner in this study was concordant with the observations of Kulsuwan and colleague, who reported that at the high MOI of the ST79 bacteriophage resulted in a better disruption of *B. pseudomallei* biofilm than low MOI[7]. Biofilm reduction by bacteriophages depends on the susceptibility of the biofilm-forming cells to the bacteriophage and to the availability of receptors for infection. The ability of bacteriophage that can encourage biofilm destruction is bacteriophage carry polysaccharide-degrading enzymes, the biofilm may be rapidly destroyed[25]. Most biofilms contain pores or water channels to allow access for the bacteriophage.

Bacteriophages 365A showed high ability to reduce ceftazidime resistant *B. pseudomallei in vitro*. However, further studies are required for the applications, even in environment or clinical applications, including *in vivo* study and it whole genome sequences. It should provide basic genetic information and could support the exactly mechanism of lysis process, toxin carrying genes, and biofilm destruction.

Conclusion

The bacteriophage 356A could be isolated from *B. pseudomallei* 365A and bacteriophage was characterized. This is the first study that has investigated the potential of bacteriophage to kill antibiotic resistant *B. pseudomallei*. Bacteriophage 365A showed high ability to reduce ceftazidime resistant *B. pseudomallei* in both planktonic and biofilm conditions. However, only bacteriophage still not effective and other study such as phage–antibiotic synergy (PAS) or modified bacteriophage engineering may provide more bacteriophage efficiency to control pathogenic bacteria. Further investigation in more detail of this bacteriophage will help us to reveal genomic and more information whether it contains virulence factors or toxins that may affect the application in the future.

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