LYTIC CAPABILITY OF BACTERIOPHAGES (FAMILY MYOVIRIDAE) ON *BURKHOLDERIA PSEUDOMALLEI*

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Abstract. *Burkholderia pseudomallei*, a gram-negative bacillus found in soil and water, is the causative agent of melioidosis. It can produce a biofilm, which increases resistance to antibacterial agents. Bacteriophages (phages) have been suggested as alternative antibacterial agents. In this study, the ability of six phages (family Myoviridae) to lyse *B. pseudomallei* isolates was examined using a microplate phage virulence assay. The six phages were more efficient in lysing soil than clinical *B. pseudomallei* isolates. Phage ST79 had the highest lytic capability, independent of inoculating phage quantity with a 4-log reduction of bacterial numbers after a 4 hour treatment. Three modified derivatives of ST79 were developed by multiple passages on phage-resistant *B. pseudomallei* isolates, leading to an increase in lytic capability from 62% to 80%. Phage ST79 at a multiplicity of infection (MOI) of 10 significantly reduces biofilm formation determined by a colorimetric method. The recovery of *B. pseudomallei* growth following phage treatment needs to be overcome if these lytic phages are to be used as biocontrol agents of *B. pseudomallei* in the environment.

Keywords: *Burkholderia pseudomallei*, bacteriophage, biofilm

INTRODUCTION

Burkholderia pseudomallei is the causative agent of melioidosis, an infectious disease endemic in Southeast Asia and northern Australia (Currie *et al*, 2000; Leelarasamee, 2000). It is a saprophyte found in soil and surface water and has been classified by the Centers for Disease Control and Prevention as a category B bioterrorism agent (Pappas *et al*, 2006). It can infect humans and animals through direct contact with contaminated soil or water via skin abrasion or inhalation (White, 2003).

Melioidosis is a public health problem in Thailand, especially in the northeast part of the country, where it is the third most common cause of death from infectious diseases (Limmathurotsakul *et al*, 2010). Control of *B. pseudomallei* in the environment may help to decrease the incidence of the disease in endemic areas. The bacterium, however, has an intrinsic resistance to several antimicrobial agents (Cheng and Currie, 2005) and also can

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produce biofilms, which increase its tolerance to antimicrobial agents (Vorachit *et al*, 1995; Sawasdidoln *et al*, 2010). Thus, there is a need to search for methods to control and eradicate *B. pseudomallei* in the environment.

Lytic phages have been suggested as biocontrol agents (Goode *et al*, 2003; Carlton *et al*, 2005; Hagens and Loessner, 2007; Kelly *et al*, 2011). Such phages also have potential for prevention and eradication of bacterial biofilms (Silankorva *et al*, 2004; Fu *et al*, 2010; Kelly *et al*, 2012). Previously, six lytic phages active against many isolates of *B. pseudomallei* have been isolated from soil in an endemic area of northeastern Thailand and characterized (Yordpratum *et al*, 2011). If they are to be used as biocontrol agents, it is necessary to estimate the lowest ratio of phage to bacteria (multiplicity of infection) (MOI) that can cause complete lysis of each isolate. In addition, the effects of lytic phages on *B. pseudomallei* biofilm have not been studied.

Thus, this study investigated the lytic capability of six *B. pseudomallei* phages belonging to the family Myoviridae (Yordpratum *et al*, 2011) and their effects on biofilm formation of several *B. pseudomallei* isolates. The knowledge gained will be a step on the way to deploy phages for biocontrol of *B. pseudomallei* in soil.

MATERIALS AND METHODS

Bacterial strains and culture condition

One hundred isolates of *B. pseudomallei*, 71 clinical and 29 soil isolates, used in this study were obtained from the Melioidosis Research Center, Khon Kaen University, Khon Kaen, Thailand. *B. pseudomallei* P37 was used as a propagating host strain (Yordpratum *et al*, 2011). Bacterial cultures were grown to mid-log phase (10^8 CFU/ml) by adding a 1% inoculum of an overnight broth culture into a nutrient broth (Oxoid, Hampshire, UK) and incubating with shaking (Innova incubator; New Brunswick, Edison, NJ) at 200 rpm, 37ºC for 4 hours. *B. pseudomallei* isolates H1038, 844, and H777 previously characterized as having low, moderate and high capability, respectively for producing biofilm (Sawasdidoln *et al*, 2010) were selected for investigation the effect of bacteriophages on biofilm formation.

Bacteriophages propagation and cocktail preparation

Six lytic phages (ST2, ST7, ST70, ST79, ST88 and ST96), previously isolated from soil in northeastern Thailand (Yordpratum *et al*, 2011), were propagated in *B. pseudomallei* P37 by the broth lysis method (Kutter and Sulakvelidze, 2005; Yordpratum *et al*, 2011). In brief, phage suspension was added at multiplicity of infection (MOI) of 0.1 to a mid-log phase culture of *B. pseudomallei* P37 in 100 ml nutrient broth containing 400 μg/ml $CaCl_2$ and incubated at 37ºC with shaking at 200 rpm until complete lysis. Bacterial debris was pelleted by centrifugation at 4,000*g*, 4ºC for 30 minutes and the supernatant filtered through a 0.22 μm membrane (Sartorius Stedim, Göttingen, Germany). Phage titer was determined using a plaque assay (Kutter and Sulakvelidze, 2005). A phage cocktail was prepared by mixing together 1 ml (10^9 PFU) of each of the six phages and storing at 4ºC until used. Another cocktail was prepared containing the addition of three modified forms of phage ST79 (see below).

Microplate phage virulence assay

Susceptibility of *B. pseudomallei* isolates to the six lytic phages was determined using a microplate phage virulence assay (Niu *et al*, 2009). In brief, a 20 μl

aliquot of each phage (2×10^8 PFU) was serially diluted 10-fold in 180 µl of nutrient broth (Oxoid, Hamshire, UK) containing 400 µg/ml $CaCl_2$ (NB/$CaCl_2$) in 96-well microtiter plate. A 20 µl aliquot of mid-log phase culture of each *B. pseudomallei* isolate (2×10^6 CFU) was inoculated into each well and the plate was incubated at 37°C for 5 hours. As a negative control, NB/$CaCl_2$ was inoculated with each bacterial culture; as positive control, each phage was inoculated with a susceptible bacterial strain (*B. pseudomallei* P37); and NB/$CaCl_2$-only was used as a plate sterility control. Wells were examined visually for turbidity due to bacterial growth. The highest dilution of phage that resulted in complete lysis of the bacteria is recorded as the multiplicity of infection (MOI), calculated by dividing the number of phages in the inoculum by the number of bacteria. Isolates of *B. pseudomallei* are defined as highly susceptible if $0.001 \leq MOI < 1$, moderately susceptible with $1 \leq MOI < 10$, minimally susceptible when $10 \leq MOI < 100$, and resistant with $MOI \geq 100$ to the test phage. Each experiment was performed in triplicate.

Modified phage preparation

A multiple passage method was used for the development of modified phages on previously phage-resistant bacterial isolates (Kelly *et al*, 2011). In brief, 1 ml aliquot of phage ST79 (MOI = 10) was added to a mid-log phase of each phage-resistant *B. pseudomallei* isolates grown in 10 ml of NB/$CaCl_2$ and incubated at 37°C with shaking. *B. pseudomallei* P37, in the presence or absence of phage ST79, was used as positive and negative control, respectively. When the positive control sample had become clear (indicating lysis of all bacteria), samples containing phage ST79 inoculated phage-resistant isolates were centrifuged at 4,000*g* for 30 minutes and supernatant filtered through a 0.22 µm membrane (Sartorius Stedim, Göttingen, Germany). A 1 ml aliquot of the filtered supernatant was repeatedly propagated on the same phage-resistant *B. pseudomallei* strain until the culture broth became clear, indicating that a high titer of a modified phage was obtained. Modified phages were plaque-purified, and named according to the lytic phage and the propagating host used. Their host ranges were determined by a spot assay (Cerca *et al*, 2007).

Assay of phage bactericidal activity

Bactericidal activities of phage ST79 and a cocktail of six phages were determined by a time-kill assay (Cerca *et al*, 2007). In brief, a mid-log growth phase of *B. pseudomallei* P37 was diluted to 10^8 CFU/ml in 3 ml of NB/$CaCl_2$. Phage ST79 then was added at MOI of 0.1, 1 and 10, respectively and the cocktail of six phages at MOI of 0.1, and the samples were incubated at 37°C with shaking. The numbers of bacteria remaining at 0, 2, 4, 6, 8, 12 and 24 hours using a plate count technique (Cerca *et al*, 2007). Experiments were performed twice in triplicate.

Assay of bacteriophage effect on biofilm formation

The efficacy of the six phages on *B. pseudomallei* P37 biofilm formation was determined in a 96-well polystyrene flat-bottom plate using a colorimetric method (Knezevic and Petrovic, 2008). In short, a 100 µl aliquot of mid-log phase culture of *B. pseudomallei* grown in Luria-Bertani (LB) broth was inoculated into double strength LB broth (2xLB) supplemented with 1% glucose. A 100 µl aliquot of inoculated medium (10^7 CFU) was added to each well, followed by 100 µl of phage in SM buffer [50 mM Tris HCl pH 7.5, 0.1

Table 1
Susceptibility of *B. pseudomallei* isolates to lysis by the test phages (family Myoviridae).

Phage	No of *B. pseudomallei* isolates			
	Susceptible[a]			Resistant
	High	Moderate	Low	
ST2	8	34	12	46
ST7	15	27	11	47
ST70	23	18	12	47
ST79	30	18	13	39
ST88	21	21	10	48
ST96	26	18	11	45
Cocktail of the 6 phages	32	17	13	38
Cocktail of 9 phages[b]	32	19	29	20

[a]Susceptibility to phage was classified according to the lowest ratio of phage to bacteria (MOI) that completely lysed the bacteria after exposure for 5 hours. High, $0.001 \leq MOI < 1$); moderate, $1 \leq MOI < 10$); low, $10 \leq MOI < 100$; resistant, $MOI \geq 100$. [b]Six phages and 3 modified phages.

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 hours. Fluid in each well was removed and replaced with 100 µl of phage at the same MOI and 100 µl of 2xLB. After incubation at 37°C for an additional 24 hours, plate was washed three times with 200 µl of sterilized water. The attached bacteria in each well were fixed with 250 µl of absolute methanol for 15 minutes each time, drained of fluid and dried at room temperature. Each well was stained with 200 µl of 0.4% crystal violet for 15 minutes, rinsed with running tap water and dried at room temperature. The crystal violet in each well was dissolved in 250 µl of 33% (v/v) glacial acetic acid and optical density (OD) of each well was measured at 595 nm using the microtiter plate reader (Multiskan Plus MK II; Labsystems, Helsinki, Finland). Negative controls containing 2xLB and SM buffer and untreated controls containing bacterial culture and SM buffer were included. Each experiment was performed twice in triplicate. Percent biofilm reduction is calculated from the formula: [($OD_{595\ nm}$ of untreated control - $OD_{595\ nm}$ of phage treatment)/$OD_{595\ nm}$ of untreated control] x 100.

Phage ST79 was used to determine its effect on high (*B. pseudomallei* H777), moderate (*B. pseudomallei* 844) and low (*B. pseudomallei* H1038) biofilm forming bacteria. The capability of *B. pseudomallei* to produce biofilm determined by crystal violet assay was classified according to the $OD_{595\ nm}$ as follows: low ($OD_{595\ nm} < 1$); moderate ($OD_{595\ nm} = 1\text{-}3$); and high, $OD_{595\ nm} > 3$. Phage ST79 (MOI = 10) was added immediately or after incubation of the inoculated wells at 37 °C for 3 hours or 24 hours to allow bacterial cell attachment and biofilm formation. Wells were processed as described above. After that the protocol followed the previous experiment.

Statistical analysis

All data are presented as mean ± SD. Comparisons between two groups were

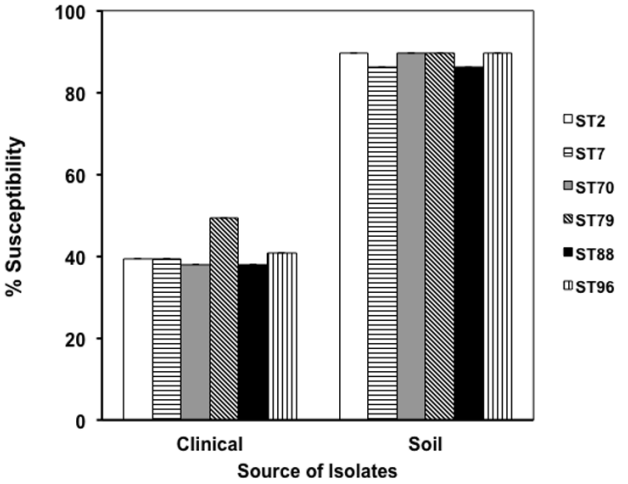

Fig 1–Percent susceptibilities of clinical and soil isolates of *B. pseudomallei* to 6 phages (family Myoviridae). A microplate phage virulence assay was used to determine the susceptibility. Results were recorded as the lowest MOI that complete lysis bacteria within 5 hours.

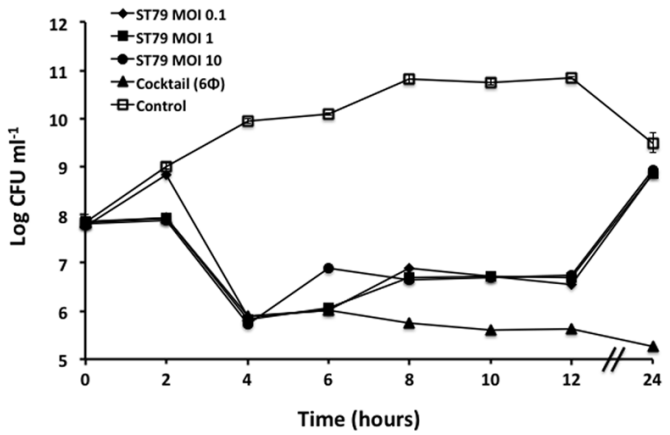

Fig 2–Bactericidal activities of phage ST79 and a phage cocktail against *B. pseudomallei* P37. Phage ST 79 at MOI of 0.1, 1 and 10 and a cocktail of 6 phages at MOI 0.1 were added to 10^6 CFU/ml *B. pseudomallei* P37. Control was a bacterium without phage infection. Results are expressed as mean ± SD.

made using the paired-samples *t*-test implemented in SPSS version 16.0 (IBM, Armonk, NY). A $p<0.05$ is considered statistically significant.

RESULTS

Lytic capability of bacteriophages against *B. pseudomallei*

Phage ST2, ST7, ST70, ST79, ST88 and ST96 lysed *B. pseudomallei* isolates by 54%, 53%, 53%, 61%, 52% and 55%, respectively (Table 1). The lowest MOIs of phages that could lyse susceptible *B. pseudomallei* isolates were in the range of 0.001 to 10. Among the susceptible group, most isolates were moderately ($1 \leq MOI < 10$) susceptible to phage ST2 and ST7, while highly ($0.001 \leq MOI < 1$) susceptible to phages ST70, ST79 and ST96. Thirty-eight isolates of *B. pseudomallei* exhibited resistance to the cocktail of six phages. The phages were more efficient at lysing soil isolates (ranging from 86%-89%) than clinical isolates (38%-49%) (Fig 1).

In order to increase lytic capability of phage against phage-resistant *B. pseudomallei* isolates, phage ST79 was modified by repeated propagation in the phage-resistant isolates. Three modified derivatives of phage ST79, namely ST79-NP18, ST79-AC9 and ST79-466, were obtained after three passages of propagation, resulting in their ability not only to lyse their previously resistant hosts but also additional 16, 6 and 5 phage-resistant *B. pseudomallei* isolates, respectively (Table 2).

The cocktail of six original phages and three derivatives of ST79 lysed 18/38 (47%) phage-resistant isolates, leading to an increase in lytic capability from 62% to

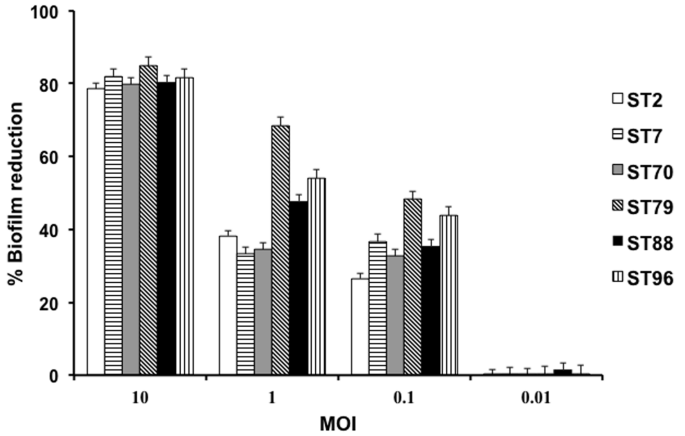

Fig 3–Effect of six phages (family Myoviridae) on *B. pseudomallei* P37 biofilm formation. Biofilm was measured by a crystal violet assay after challenge with phages at MOI of 0.01, 0.1, 1 and 10. Results are expressed as percent biofilm relative to untreated controls.

80% of the *B. pseudomallei* isolates tested (Table 1).

Phage bactericidal activity

Bactericidal activity of phage ST79 against a planktonic culture of *B. pseudomallei* P37 at different MOIs was similar (Fig 2). After 4 hours of treatment with phage, viable counts of bacteria were reduced by about a 4-log CFU/ml, corresponding to 99.99% of *B. pseudomallei* cells. However, after 12 hours the bacteria were able to regrow. A cocktail of six phages could inhibit regrowth resulting in 4-log reduction of bacterial numbers for at least 24 hours.

Effect of phages on *B. pseudomallei* biofilm formation

B. pseudomallei P37, a highly susceptible isolate with low capacity for producing biofilm was used to determine the effect of different concentrations of phages on biofilm formation compared with the untreated controls. At a high MOI (MOI = 10), phage ST2, ST7, ST70, ST79, ST88 and ST96 significantly reduces biofilm formation by 78%, 82%, 79%, 85%, 80% and 81%, respectively ($p < 0.05$) (Fig 3).

The ability of phage ST79 to reduce biofilm formation then was investigated against three isolates of *B. pseudomallei* differing in their capacity to produce biofilm, namely, H1038 (low), 844 (medium) and H777 (high). These three isolates were susceptible to phage ST79 with the lowest MOI of 0.1. The MOI of 10 was chosen to use in this study as the high MOI was more effective to reduce biofilm formation than the low MOI (Fig 3). The effects of phage ST79 at MOI of 10 on reducing *B. pseudomallei* biofilm formation were variable depending on the stage of biofilm formation prior to exposure to phage and the capacity of the different isolates to produce biofilm. If phage ST79 was applied immediately before the attachment of bacterial cells to the microplate surface, it reduced biofilm formation by 95.34 ± 0.03, 89.33 ± 0.01 and 74.52 ± 0.22% in H1038, 844 and H777 isolate, respectively. On the other hand, if the bacterial cells were allowed to adhere to the microplate surface for 3 hours, phage ST79 reduced biofilm formation of H1038, 844 and H777 isolate by 78.89 ± 0.05, 86.74 ± 0.07 and 50.21 ± 0.10%, respectively. The ability of phage ST79 to reduce *B. pseudomallei* H1038, 844 and H777 biofilm formation of 24 hours was 36.04 ± 0.06, 80.75 ± 0.04 and 27.78 ± 0.22%, respectively.

DISCUSSION

Among the six *B. pseudomallei* lytic phages (ST2, ST7, ST70, ST79, ST88 and ST96), phage ST79 was the most effective against both soil and clinical isolates.

Table 2
Lytic capability of modified phages on phage resistant *B. pseudomallei.*

B. pseudomallei isolates	Lytic capability of modified phage		
	ST79-NP18	ST79-AC9	ST79-466
409	+	-	-
466	+	+	+
10020	-	-	-
1-602	-	-	-
3-54	-	-	-
3-82	+	-	-
3-87	-	-	-
3-91	+	+	+
3-139	-	-	-
5-278	+	-	-
5-340	+	-	-
A1	+	-	-
AC9	-	+	-
AC17	+	-	-
AC18	+	-	-
MD006	-	-	-
MD009	-	-	-
MD10	+	-	-
MD14	-	+	+
MD16	-	-	-
MD18	-	-	-
MD19	-	-	-
MD22	+	+	+
MD23	-	-	-
MD25	-	-	-
MD26	-	-	-
MD33	-	-	-
MN17	-	-	-
NL001	-	-	-
NL002	+	-	-
NL004	-	-	-
NL17	-	-	-
NP18	+	-	-
NP27	-	-	-
UT5	-	-	-
UT4	+	-	-
UT6	+	-	-
UT7	+	+	+
	16	6	5

+, clear plaque formation, sensitive; -, no plaque formation, resistant.

However, a cocktail of the six phages did not increase lytic capability suggesting that the six phages had similar activity against the tested strains. The resistance to phages exhibited by 38 *B. pseudomallei* isolates may be due to such factors as variations of receptors, adsorption blocking, intracellular restriction modification system or abortive infection which limits phage replication within a bacterial population (Labrie *et al*, 2010). Another possible mechanism responsible for phage resistance phenotype is lysogeny, by which phage genome integrated into host's genome (Abedon, 2006). Resistance conferred by the restriction modification system can sometimes be overcome if the phage is passaged multiple times through the phage- resistant bacteria (O'Flaherty *et al*, 2005; Kelly *et al*, 2011). In this study, three modified derivatives of phage ST79 were developed that could lyse 18 of the 38 previously phage resistant *B. pseudomallei* isolates, suggesting that resistance in these isolates might be due to the restriction modification system. According to a previous study (Yordpratum *et al*, 2011), the receptors of *B. pseudomallei* lytic phages are lipopolysaccharides, which are heterogeneous in structure (Anuntagool *et al*, 2006), suggesting another possible mechanism of phage resistance.

The bactericidal activities of phage ST79 at different MOIs against the susceptible *B. pseudomallei* isolates were similar suggesting that its lytic activity did not depend on the initial phage quantity. However, after 12 hours *B. pseudomallei* was able to regrow, possibly due to the appearance of a resistant mutant or possibly bacteria carrying lyogenic phage. The cocktail of six phages could inhibit the regrowth (for at least 24 hours) suggesting that a phage cocktail should be used instead of a single phage type. Similarly, phage cocktails reduce phage-resistant mutants of *E. coli* O 157:H7 (O'Flynn *et al*, 2004) and *Klebsiella pneumonia* (Gu *et al*, 2012).

The observed reduction of *B. pseudomallei* biofilm in a dose dependent manner in this study (Fig 3) agrees with the observations of Corbin *et al* (2001), who reported that increasing the MOI of the T4 phage by 100-fold resulted in a greater disruption of *E. coli* biofilm than a 10-fold increase. Biofilm reduction by phages depends on the susceptibility of the biofilm-forming cells to the phage and to the availability of receptors for infection. If the phage also possesses polysaccharide-degrading enzymes, the biofilm may be rapidly destroyed (Hughes *et al*, 1998). Most biofilms contain pores or water channels to permit access for the phage (Sutherland *et al*, 2004). Thus phage ST79 was capable of penetrating the biofilm to gain access to the bacteria and causing lytic cell death. In this study, the efficiency of phage ST79 to reduce biofilm formation of *B. pseudomallei* was dependent on the amount of biofilm produced by each isolate and the period of attachment to the surface of microtiter plate. It was able to reduce greatly the biofilm (74.52%-95.34%), if treated immediately. This can be explained that initial bacterial attachment which is the first step in biofilm formation is reduced resulting in biofilm reduction. Similarly, significant biofilm reduction was achieved within 24 hours of phage treatment against *Pseudomonas aeruginosa* (Knezevic and Petrovic, 2008). The ability of phage ST79 to reduce *B. pseudomallei* biofilm formation after 3 hours attachment was more than 50% (50.14%-86.74%) suggesting that at the early stage of biofilm formation, phage may be able to diffuse through matrix to reach bacterial cells. Similar result has been reported for *Pseudomonas fluorescens*

biofilm formation, which is reduced by up to 80% in the early stage of biofilm development (Silankorva *et al*, 2004). On the other hand, the effect of phage on established biofilm (24 hours) was diminished in H1038 (36.04%) and H777 (27.78%) suggesting that the physiological state of biofilms may affect the lytic performance of the phage. The subsequent reduction in biofilm formation (after 24 hours) is the result combination of the initial amount of biofilm, the activity of the released biofilm degrading enzyme(s) and the numbers of bacterial cells at the time of assay. The high ability of phage for reducing biofilm of *B. pseudomallei* isolate 844 following 3 hours (86.74%) and 24 hours (80.75%) of attachment may be due to the difference in the biofilm structure of this isolate that allows the phage access to the biofilm interior and lyse the susceptible cells.

In conclusion, all 6 *B. pseudomallei* phages (family Myoviridae), particularly phage ST79, have the lytic capability against both planktonic and biofilm-forming forms of *B. pseudomallei*. Resistance to phage infection could be overcome in some *B. pseudomallei* isolates by a number of repeated infection. A cocktail of phages prevented regrowth of phage-treated *B. pseudomallei*, but for how long needs further investigation if phages are to be employed as biocontrol agents of *B. pseudomallei* in the environment.

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