Identification of the *Burkholderia pseudomallei* bacteriophage ST79 lysis gene cassette

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Abstract

**Aims:** To identify and characterize the lysis gene cassette from the bacteriophage ST79 that lyse *Burkholderia pseudomallei*.

**Methods and Results:** Approximately 1-5 kb of ST79 lysis genes were identified from the phage genome data. It was composed of *holin, peptidase M15A or endolysin, lysB* and *lysC*. Each gene and its combinations were cloned into *Escherichia coli* and the lytic effects were measured. Co-expression of *holin* and *peptidase M15A* showed the highest lysis activity. Expression of *holin, lysB/C* or *holin-peptidase M15A-lysB/lysC* lysed the *E. coli* membrane, whereas *peptidase M15A* alone did not. The predicted transmembrane structures of *holin* and *lysB/C* indicated that they could be inserted into the bacterial membrane to form pores, affecting cell permeability and causing lysis.

**Conclusion:** This is the first report of an investigation into the lysis genes of *B. pseudomallei*’s lytic phage using *E. coli* as a model.

**Significance and Impact of the Study:** *Burkholderia pseudomallei*, a Gram-negative bacterium causing an infectious disease, is intrinsically resistant to several antibiotics, and a vaccine is not available. The lysis genes of ST79, the first reported lytic bacteriophage of *B. pseudomallei*, were characterized. The development of ST79 as an alternative treatment for skin ulceration, for example, or to be used as a gene cloning tool for *B. pseudomallei* may be possible with this knowledge.

Introduction

*Burkholderia pseudomallei*, a Gram-negative, Beta-proteobacterium living in soil and water, causes a severe infectious disease known as melioidosis. It infects humans and animals through skin cuts, inhalation or ingestion of contaminated soil and water (Currie et al. 2008). The pathogen is intrinsically resistant to many antibiotics such as penicillin, first- and second-generation cephalosporins, macrolides, rifamycins and aminoglycosides (Cheng and Currie 2005) and requires long-term use of third-generation antibiotics to avoid relapse, which increases treatment costs substantially (Lipsitz et al., 2012). Because, to date, there is no vaccine available in the market (Limmathurotskul et al. 2015), the importance of battling drug-resistant isolates that may arise in the near future to prevent sepsis associated with a high mortality rate, alternative treatments or adjunct therapies for *B. pseudomallei* needs to be emphasized.

Bacteriophages or phages are viruses that infect bacteria. They can be found wherever their hosts are present in fresh water, seawater, sediment, soil, deep sea vents, sewage and faeces (Bergh et al. 1989; Danovaro and Serresi 2000; Danovaro et al. 2001; Hewson et al. 2001). After reproducing inside the cell, the bacteriophage must exit from its bacterial host cell to release progenies and
B. pseudomallei bacteriophage lysis genes

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disperse to new cells. There are two different strategies depending on the phage genome type. Single-stranded DNA phages use a single lysis gene such as the lysis protein E of the ΦX174 phage (Witte et al. 1992) or secreted via their own secretion system such as the M13 phage of Escherichia coli (Rakonjac et al. 1999), while double-stranded DNA phages use two proteins holin, a transmembrane protein which inserts itself into the bacterial inner membrane, and the endolysin, a small murelalytic enzyme that cleaves the bacterial peptidoglycan to release phage progenies (Young 1992). Moreover, lytic accessory genes were also reported in phages found in Gram-negative bacteria such as Rz/Rz1 in the lambda phage and PRD1 (Berry et al. 2008; Kvitko et al. 2013) or lysB/C in the P2 phage (Ziermann et al. 1994) that help during the lysis process.

The direct use of a phage to kill pathogenic bacteria on skin ulcers and wounds has been reported in a few experiments, such as the treatment of E. coli, Proteus spp., Pseudomonas spp. and Staphylococcus spp., by a combination of lytic bacteriophages impregnated with an antibiotic in a biodegradable polymer (Markoishvili et al. 2002). Results showed that a success rate of 70% in 22 patients stimulated the possibility for further investigations in other bacteria. Before a phage particle can be accepted for the use in human therapy, even though in in vitro, the phage genome and its lytic enzymes require full characterization.

Previously, our laboratory surveyed for the presence of lytic phages in 140 soil samples from northeast Thailand, an area that has one of the highest incidences of melioidosis cases in the world (Yordpratum et al. 2011). Six novel bacteriophages, namely ST2, ST7, ST70, ST79, ST88 and ST96, were found to lyse B. pseudomallei. Among these, bacteriophage ST79 was the most effective in lysing a wide range of B. pseudomallei strains and also B. mallei, but it did not cause lysis of the closely related B. thailandensis and other nonpathogenic bacteria (Yordpratum et al. 2011). Full genome sequence analysis of bacteriophage ST79 did not show the presence of known toxins and integrases that might be harmful to humans (manuscript in preparation). In this study, it was aimed to: (i) express and characterize the ST79 lysis gene cassette in E. coli, (ii) perform a comparison with genes present in other bacteriophages, and (iii) identify the lysis activity using E. coli as a model.

Materials and methods

Bacteriophages and bacteria

The bacteriophages ST79 was isolated from soil samples in the Khon Kaen province, Thailand (Yordpratum et al. 2011). The Burkholderia pseudomallei strain P37 was isolated from the blood of a patient admitted to Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand. The strain was used for B. pseudomallei lytic phage propagation. The Escherichia coli strain LMG194 was purchased from Invitrogen (Grand Island, NY) and used as a host for all cloning and protein expression procedures. Because the expression of lysis genes requires a low copy number plasmid and strictly controlled conditions to prevent the host cell from lysis before the induction, the E. coli strain LMG194 was used as a model to test for the activity of each lysis gene construction.

Bioinformatics analysis of ST79 lysis gene cassette and primer design

The draft of the phage ST79 DNA sequence was used for comparisons against the GenBank database (BLASTN and BLASTP searches). It showed very high homology with the prophage found in the Burkholderia glumae BGR1 genome (Lim et al. 2009). Nucleotide sequences predicted to be phage lysis genes were used to design specific primers for amplification of each gene by PCR (Table 1). Oligonucleotides were also designed to contain different restriction enzyme digestion sites to assist with cloning. The nucleotide identity search was done with BLASTN and annotation of the predicted open reading frames (ORF) of the ST79 genome was performed using BLASTP in the NCBI database (http://www.ncbi.nlm.nih.gov/) (manuscript in preparation). The analysis of the ST79 full-length genome provided putative lysis gene sequences (GenBank ID: 509141608). The SOSUI software ver. 1.11 (Hirokawa et al. 1998) was used to predict the secondary structure of protein transmembrane regions, while the INTERPROSCAN software ver. 4.8 (Zdobnov and Apweiler 2001) was used to analyze protein functions. EMBOSS (The European Molecular Biology Open Software Suite) (Rice et al. 2000) was used for cleavage site prediction on a protein sequence.

Phage propagation and DNA extraction

The ST79 phage was propagated in a liquid culture and DNA was extracted as described elsewhere (Sambrook and Russell 2001) with some modifications. Briefly, 1% inoculum of an overnight culture of B. pseudomallei P37 was inoculated into 100 ml of fresh nutrient broth and incubated at 37°C with shaking for 4 h to reach its mid-log phase that was found to be suitable for the phage infection. Then, a purified ST79 phage suspension (Multiplicity of Infection (MOI) = 0.1) and 400 µg ml⁻¹ of CaCl₂ were added. The culture was further incubated at 37°C for 3–5 h to obtain complete lysis of the bacteria,
followed by centrifugation at 2000 g at 4°C for 30 min to remove the bacterial cell debris. The phage suspension was filtered through a 0.22-μm membrane and precipitated by 10% polyethylene glycol (PEG, MW 8000) at 4°C overnight. The precipitated phage was collected by centrifugation at 2000 g for 30 min (rotor J-14, Beckman, Brea, CA) and resuspended with 500 μl of SM buffer (50 mmol l\(^{-1}\) Tris-HCl, pH 7.5, 100 mmol l\(^{-1}\) NaCl, 10 mmol l\(^{-1}\) MgSO\(_4\), and 0.01% gelatin solution). Bacterial nucleic acids were digested with DNase I (5U) for 30 min (rotor J-14, Beckman, Brea, CA) and the phage particles were lysed by adding 0.5 g l\(^{-1}\) of SDS and 0.073 g l\(^{-1}\) of EDTA and incubated at 65°C for 15 min. The phage genomic DNA was extracted with the phenol-chloroform method and then dissolved in 50 μl TE buffer.

Cloning of the ST79 lysis genes

The predicted lysis genes were cloned into the expression vector, pBAD/HisA (Invitrogen), following Lim et al. (2012) and Green and Sambrook (Vol 1, 2012) with some modifications. Lysis genes were amplified by PCR using ST79 genomic DNA as the template. Each single gene, holin, peptidase M15A and lysB/C, was amplified using specific primers (Table 1) and cloned into the plasmid pBAD/HisA using the KpnI and SacI restriction sites. The combinations of genes holin-peptidase M15A-lysB/C (whole lysis cassette), holin-peptidase M15A and peptidase M15A-lysB/C were amplified by the upstream gene forward and downstream gene reverse primers, and cloned using KpnI and SacI restriction sites. For the holin and lysB/C gene combinations, they were ligated using the Smal restriction site before being cloned into pBAD/HisA as previously described. PCR products were purified with the QiAquick PCR Purification kit (Qiagen Inc., Hilden, Germany), cloned into the pBAD/HisA expression vector and transformed into *E. coli*. The correct insert size was confirmed and selected clones were sequenced at the University of Texas, Genomics Core Facility (Arlington, TX).

Protein expression analysis by Western blot

Transformed cells of *E. coli* strain LMG194, each containing a recombinant plasmid with lysis genes, were grown at 37°C with shaking until mid-exponential phase. Cells were induced by addition of 0.2% L-arabinose for 4 h and the pellets were harvested by centrifugation for further analysis. The 15 μg of total proteins were mixed with SDS sample buffer and boiled before being loaded on 15% SDS-PAGE (Sambrook and Russell 2001). Proteins from each clone were resolved on SDS-PAGE, stained with commassie brilliant blue R250 and blotted onto a polyvinylidene difluoride (PVDF) membrane using the semi-dry blotting system (BioRad Laboratories Inc., Hercules, CA). For detection, Penta-His Mouse IgG1 monoclonal antibody (cat.no. P21315) (Thermo Fisher Scientific, Waltham, MA) and polyclonal rabbit anti-
mouse IgG-HRP antibody (cat.no. P016102) (Dako, Denmark) were used as the primary and secondary antibody, respectively, before the chemiluminescence (ECL) signal was generated using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Signal detection was performed with a ImageQuant LAS4000 Luminescent Image Analyzer (Amersham Biosciences, Buckinghamshire, UK) (Green and Sambrook 2012).

The lysis activity of phage enzymes expressed in *Escherichia coli*

The lysis protein induction and expression procedures were done as described by Lim et al. (2012) with some modifications. *Escherichia coli* LMG194 cells containing the recombinant plasmid pBAD/HisA-holin-peptidase M15A-lysB/C were inoculated into LB broth supplemented with 100 µg ml⁻¹ of ampicillin and 40 µg ml⁻¹ of tetracycline and were grown at 37°C for 16–18 h with shaking. One percent of the overnight cultures were inoculated into LB broth and grown until the mid-exponential phase (OD₆₀₀ = 0.5–0.6). L-arabinose (0.2%) was added to the cultures and 1 ml was collected every hour to measure at optical density (OD₆₀₀) using disposable cuvette (BRAND in Wertheim, Germany) and the Spectronic 20D+ spectrophotometer (Thermo Scientific). The *E. coli* LMG194 containing holin-peptidase M15A-lysB/C plasmid without the addition of 0.2% L-arabinose and noninduced *E. coli* LMG194 containing pBAD/HisA were used as negative controls. Bacterial growth curves are the means of three independent experiments.

For other plasmid constructions, the expressions of pBAD/HisA-holin, pBAD/HisA-peptidase M15A, pBAD/HisA-lysB/C, pBAD/HisA-holin-peptidase M15A, pBAD/HisA-peptidase M15A-lysB/C, and pBAD/HisA-holin-lysB/C, were performed as previously described, but 1 ml of the bacterial cultures was taken every 20 min for OD₆₀₀ measurements.

Results

The gene structure of the ST79 lysis cassette and genes sequence analyses

The *in silico* analysis of ST79 genome sequence (GenBank ID: 509141608) showed the presence of a putative lysis cassette, containing four genes spanning approx. 1.5 kb in size. The cassette encoded a hypothetical protein (135 codons), peptidase M15A (150 codons), LysB (147 codons) and LysC (98 codons) with a 36 base space between the hypothetical protein and the peptidase M15A (Fig. 1). The start codon of the lysB gene was overlapped with the stop codon of peptidase M15A and the first 54 codons of lysC were embedded in the lysB-coding region (Fig. 1).

When the hypothetical protein was analyzed using BLASTP, it showed a putative conserved domain FlaA1 (COG1086) of predicted nucleoside-diphosphate sugar epimerases (cell envelope biogenesis, outer membrane/carbohydrate transport and metabolism) (E-value = 4.51e⁻⁰³) at the interval amino acid position 14–28. The hypothetical protein showed 100% similarity (E-value = 8e⁻⁸⁷) with a *Burkholderia glumae* BGR1 hypothetical protein located in its prophage region and other bacterial hypothetical proteins as shown in Table 2. The SOSUI prediction software indicated three transmembrane regions similar to the holin type I (Wang et al. 2000) (Fig. 2).
The peptidase M15A protein showed a high similarity (68–98%) with the peptidase M15 and endolysin of other phages found in the genus Burkholderia and the peptidase M15_3 superfamily conserved domain was localized to amino acids 6 to 121 (PF08291) (E-value 7.48e-31). This also appeared with LysB/C that had a transmembrane domain and predicted to have cleavage form of the transmembrane protein as predicted by EMBOSS (score 7.5). This also appeared with LysB/C that had a transmembrane domain and predicted to have cleavage sites to obtain a product of approx. 19 kDa that appeared in all lanes (2–5) (Fig. 3). The lower band of approx. 8–10 kDa that appeared together with holin may be a cleavage form of the transmembrane protein as predicted by EMBL (score 7-5). This also appeared with LysB/C that had a transmembrane domain and predicted to have cleavage sites to obtain a product of approx. 17 kDa (score of 6-5) (Fig. 3, lane 6). The expressed products of lysB/C (20–3 kDa) and peptidase M15A (20–6 kDa) that were constructed as the first upstream gene and linked to His-tag had molecular weight of approx. 19 kDa that appeared in all lanes (2–5) (Fig. 3). The lower band of approx. 8–10 kDa that appeared together with holin may be a cleavage form of the transmembrane protein as predicted by EMBL (score 7-5). This also appeared with LysB/C that had a transmembrane domain and predicted to have cleavage sites to obtain a product of approx. 17 kDa (score of 6-5) (Fig. 3, lane 6). The expressed products of lysB/C (20–3 kDa) and peptidase M15A (20–6 kDa) that were constructed as the first upstream gene and linked to His-tag had molecular weight of approx. 19 kDa that appeared in all lanes (2–5) (Fig. 3). The lower band of approx. 8–10 kDa that appeared together with holin may be a cleavage form of the transmembrane protein as predicted by EMBL (score 7-5).
plasmids grew to an OD600 of approx. 1 observed (Fig. 5). Cells containing only the peptidase E. coli in -peptidase M15A did not lyse, while cultures containing the genes model. construction and also the expression was under E. coli 0 decreases at OD600. Moderate lysis was observed when for cell lysis with a decrease in OD600 from 0 pBAD/HisA-lysB/C ST79 (5), pBAD/HisA-peptidase M15A (8) and protein size marker (M).

Figure 2 Bioinformatic analysis of ST79 lysis protein sequences. The amino acid sequences are arranged from N-terminal to C-terminal. Interproscan of domain analysis, labelled in grey colour, indicating the Hedgehog signalling/DD-peptidase zinc-binding domain that was found in peptidase M15A sequence and the signal peptide domain that was found at the N-terminal of both lysB and lysC. The transmembrane domain predictions by Sosui showed three domains in hypothetical protein (Holin) and one in the lysB protein sequence (underlined amino acid).

Figure 3 Western blot detection of protein products expressed from recombinant lysis genes in Escherichia coli using Penta-His Mouse IgG1 antibody. Protein products after 4 h induction from E. coli containing pBAD/HisA (1), pBAD/HisA-holin (2), pBAD/HisA-holin-peptidase M15A (3), pBAD/HisA-holin-lysB/C (4), the whole lysis cassette of ST79 (5), pBAD/HisA-lysB/C (6), pBAD/HisA-peptidase M15A (7), pBAD/HisA-peptidase M15A-lysB/C (8) and protein size marker (M).

decreased from 0.5 to below 0.1 within 1 h after induction, while E. coli that contained noninduced control pBAD/HisA and pBAD/HisA-holin-peptidase M15A-lysB/C plasmids grew to an OD600 of approx. 1.0 (Fig. 4).

When lysis genes in different constructs were induced in E. coli LMG194 at OD 600 = 0.6, cell lysis was observed (Fig. 5). Cells containing only the peptidase M15A did not lyse, while cultures containing the genes peptidase M15A-lysB/lysC and lysB/lysC showed slight decreases at OD600. Moderate lysis was observed when constructs for the expression of holin and holin-lysB/C were used. The construct combination containing the genes for holin and peptidase M15A was the most effective for cell lysis with a decrease in OD600 from 0.6 to below 0.1. This effect, however, can be influenced from different construction and also the expression was under E. coli model.

Discussion

In this study, it was determined that the gene arrangement of the whole lysis cassette in the ST79 phage genome is similar to other phage genomes observed in Gram-negative bacteria, including the λ phage model. In general, the cassette is organized in the following order: holin, endolysin, lysB and lysC (Rz and Rz1) (Summer et al. 2007). The approx. 1.5 kb of ST79 lysis cassette showed 97% identity with the prophage present in the B. glumae BGR1 genome. This species is known as a causative agent of grain and seedling rot in rice or named as bacterial wilt in many field crops (Kim et al. 2004). The prophage was suspected to influence the bacterial virulence during plant disease (Varani et al. 2013). Even though B. glumae has not yet been reported in Thailand, the high identity shared between the ST79 lysis genes and those observed in the prophage of B. glumae may suggest the possibility of the transference of the phage between these two bacterial species.

The organization of ST79 lysis genes consisted of a hypothetical protein, peptidase M15A, lysB and lysC. This is similar to the approx. 1.1 kb lysis cassette found in the genome of Pseudomonas aeruginosa phage ΦKMW containing KMV44, 45, 46 and 46-1 genes encoding for holin, endolysin, Rz/Rz1 (Briers et al. 2011). The ST79 lysB/lysC genes belong to the Rz/Rz1 overlapping class that is similar to what has been reported in Enterobacteria phage P2, PsP3, Wphi and Burkholderia phage ΦE202. All of them differ from Gram-negative phage, PRD1, that contains the same lysis genes but with a different organization (VII–XXXV–XXXVI and XXXVII genes) composed of endolysin, holin, Rz and Rz1 (Krupovic et al. 2008). Therefore, the hypothetical gene in the ST79 phage was concluded to be holin.

BLASTP comparisons of each lysis gene in ST79 revealed the high identity of peptidase M15A (68–98%) and LysB (90–99%) with other Burkholderia spp. Holin and lysC, however, showed identity to hypothetical protein in other
Burkholderia spp. These genes may be annotated later to be a part of lysis cassette. For nucleotide sequence similarity among lytic phages of B. pseudomallei, Southern blot hybridization using peptidase M15A sequence of ST79 as a probe only hybridized with ST7 but not with the ST70, 88, 96 and F4. The nucleotide sequence of lysis ST79 as a probe only hybridized with ST7 but not with their progeny from the bacterial host. The classic holin-endolysin system has been reported in several phages of Gram-positive and Gram-negative bacteria (Wang et al. 2000; Young et al. 2000). For phages found in Gram-negative bacteria, there are two genes encoding the auxiliary lysis proteins Rz and Rz1 (Young et al. 2000) or lysB/lysC equivalents (Summer et al. 2007). The λ phage lysis genes have been extensively studied for the lysis mechanism. In standard laboratory conditions, the holin and endolysin are sufficient for the lysis and release of the progeny virions, but under high concentrations of divalent cations, the Rz/Rz1 are necessary for effective lysis (Young et al. 1979; Bläsi et al. 1999). Krupovic and coworkers reported that the Rz/Rz1-like accessory lysis genes of bacteriophage PRD1 are required for full lysis as the lysis rate decreases in the PRD1 Rz/Rz1-deficient phage when divalent cations are absent (Krupovic et al. 2008). In the presence of 10 mmol L⁻¹ MgCl₂, the lysis rate was dramatically reduced. The absence of Rz/Rz1, encoded by gene 15 of P22 bacteriophage caused the decrease in plating efficiency by 3 to 6 logs in the presence of divalent cations (Casjens et al. 1989). Nevertheless, Jeong-A Lim et al. (2012) reported that for a Gram-negative lysis system, the lysis mechanism of Salmonella typhimurium-infecting bacteriophage, SPN1S, that required only endolysin and Rz/Rz1-like proteins is uncommon.
In this present work, the lysis activities of each lysis gene and combination of ST79 phage were observed in E. coli. The co-expression of the holin and peptidase M15A genes was sufficient to lyse E. coli from the inside out. When holin alone or its combination with either peptidase M15A or lysB/lysC were used, a moderate cell lysis was observed. This was not the case, when peptidase M15A was expressed alone. This phenomenon of holin-dependent translocation of endolysin is consistent with λ and the T4 phage lysis mechanism (Gründling et al. 2001; Wang 2006). The transmembrane structure of holin was predicted to function by causing an accumulation of proteins in the cytoplasmic membrane (CM) and triggering the disruption of the CM to release the active peptidase M15A from cytosol. This peptidase gains access to ing the disruption of the CM to release the active pept-
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nism of SAR before the endolysin can have access to pep-
tidoglycan (Park et al. 2011). The signal anchor-release (SAR) in the sec secretory system allows the endolysin to be exported into the periplasmic space, requiring the action of (pin) holins to form holes and activation of the depolarization mechanism of SAR before the endolysin can have access to pep-
tidoglycan (Park et al. 2007; Pang et al. 2009).

The lysB or Rz protein structure was predicted to contain a single transmembrane domain (TMD), which could insert itself into the inner membrane, while the lysC or Rz1 was predicted to encode a lipoprotein to act on the outer membrane (Summer et al. 2007). According to the domain prediction and lysis function on the bacterial membrane, the lysB/lysC protein complex of ST79 has a similar topology as the Rz/Rz1 of λ phage (Berry et al. 2008) and protein P36/P37 of the PRD1 phage (Krupovic et al. 2008). Present results showed that this complex caused a slight lysis when expressed, but was not as effective as holin. It is noteworthy to mention that the lysis activity when the four genes were expressed was not as effective as the holin-peptidase M15A con-
struct. It is therefore hypothesized that the E. coli and B. pseudomallei cytoplasmic membranes may have specific differences that are required for an efficient lysis by the ST79 gene cassette or the outcome was influenced by the gene constructed for recombinant protein expres-
sion.

In conclusion, this is the first report of lysis genes and their lysis activities from lytic B. pseudomallei phage, ST79, that was investigated in an E. coli model. The basic knowledge might lead to further study using the ST79 phage as an alternative treatment locally, especially in diabetic wounds or for the development of ST79 as a molecular biology tool for cloning of B. pseudomallei.

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Conflict of Interest

No conflict of interest declared.

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