Functional redundancy of *Burkholderia pseudomallei* phospholipase C enzymes and their role in virulence

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Phospholipase C (PLC) enzymes are key virulence factors in several pathogenic bacteria. *Burkholderia pseudomallei*, the causative agent of melioidosis, possesses at least three plc genes (*plc1*, *plc2* and *plc3*). We found that in culture medium *plc1* gene expression increased with increasing pH, whilst expression of the *plc3* gene was pH (4.5 to 9.0) independent. Expression of the *plc2* gene was not detected in culture medium. All three *plc* genes were expressed during macrophage infection by *B. pseudomallei* K96243. Comparing *B. pseudomallei* wild-type with *plc* mutants revealed that *plc2*, *plc1* or *plc3* mutants showed reduced intracellular survival in macrophages and reduced plaque formation in HeLa cells. However, *plc1* or *plc3* mutants showed no significant differences in plaque formation compared to wild-type bacteria. These findings suggest that Plc2, but not Plc1 or Plc3 are required for infection of host cells. In *Galleria mellonella*, *plc1*, *plc2* or *plc3* mutants were not attenuated compared to the wild-type strain, but multiple *plc* mutants showed reduced virulence. These findings indicate functional redundancy of the *B. pseudomallei* phospholipases in virulence.

*B. pseudomallei*, a Gram-negative facultative intracellular bacterium, is the etiological agent of melioidosis in humans and in animals. Melioidosis in humans was at one time thought to be largely restricted to Southeast Asia and Northern Australia, but it is now thought to occur in many tropical and sub-tropical regions of the world¹. The annual global burden of meliodosis is estimated to be 165,000 cases with 89,000 deaths from the disease¹. A feature of *B. pseudomallei* is its ability to modulate a range of host-cell responses and to evade phagocyte killing activity²,³. *B. pseudomallei* has evolved mechanisms to evade phagocyte activities, including escape from phagosomes and entry into host cell cytosol where it multiplies and forms actin tails allowing cell-to-cell spreading⁴. This complex intracellular lifestyle is contributed by several bacterial virulence factors including type three secretion systems (T3SS), type six secretion systems (T6SS), polysaccharide capsule, lipopolysaccharide (LPS), and various secreted effector proteins⁵. Additionally, *B. pseudomallei* can produce many enzymes which play roles in virulence including proteases, catalase, peroxidase, superoxide dismutase¹, and phospholipase C (Plc) enzymes⁶. Plc enzymes play roles in the pathogenesis of several Gram-positive and Gram-negative bacterial infections including those caused by *Mycobacterium tuberculosis*⁷, *Pseudomonas aeruginosa*⁸, *Clostridium perfringens*⁹, *Listeria monocytogenes*¹⁰, and *Legionella pneumophila*¹¹. Also, different of Plcs play different roles in virulence¹², including tissue colonization, evasion of host defense mechanisms, escape from host cell phagosomes and/or the induction of mediators of inflammation¹³.

Analysis of the *B. pseudomallei* K96243 genome reveals genes encoding three Plc enzymes (*Plc1*, *Plc2* and *Plc3*). The genes encoding *Plc1* (*bpsl2403*) and *Plc2* (*bpsl0338*) are located on chromosome 1. These encoded proteins are predicted to be acidic, have the ability to hydrolyze phospholipids including phosphatidylcholine and sphingomyelin and are non-hemolytic¹⁴. The gene encoding *Plc3* (*bpsl0067*) is located on chromosome 2².
At present, the conditions under which the plc genes are expressed, and their roles in virulence are poorly understood. We have previously characterized the *B. pseudomallei* Plc1 and Plc2 and found that Plc2 was cytoxic\(^\text{15}\). Additionally, these Plc enzymes appear to play a role in nutrient acquisition\(^\text{14}\). Subsequently, Burtnick et al.\(^\text{15}\) showed that *B. pseudomallei* Plc1 and Plc2 are secreted from the bacterial cell via the type II secretion system (T2SS) in a GspD-dependent manner\(^\text{15}\).

Little is known about the *B. pseudomallei* Plc3 enzyme. Whole-genome microarrays have revealed that plc3 is up-regulated in vivo, and in hamsters a plc3 mutant shows reduced virulence compared to the wild-type, suggesting that it is required for virulence\(^\text{16}\). However, the mechanisms underlying attenuation are unknown. Dowling et al.\(^\text{17}\) reported that Plc3 might be a potential candidate vaccine requiring further study.

In this study, the expression of the plc genes in culture medium and in J774A.1 macrophage-like cells was analyzed using RT-PCR. *B. pseudomallei* plc1, plc2 or plc3 single mutants and plc12 or plc123 mutants were assessed for virulence in macrophages and in *Galleria mellonella* larvae. Our work provides new insights into the role of Plc enzymes in the pathogenesis of disease caused by *B. pseudomallei*.

**Results and discussion**

**Culture pH differentially affects plc1 gene expression.** *B. pseudomallei* is an intracellular bacterium. After phagocytosis by phagocytes, the bacteria encounter acidic condition within the phagosome, before escaping to survive in the cytosol\(^\text{7}\). To determine the effect of pH on the expression of the plc1-3 genes, *B. pseudomallei* K96243 was incubated in LB broth which had been adjusted to pH 4.5, 5.0, 7.0, 8.0 or 9.0 before bacterial mRNA was extracted and tested. Using RT-PCR we first showed that expression of *B. pseudomallei* 23S RNA was similar at all pH conditions tested. RT-PCR revealed that the level of transcription of the plc1 gene increased between pH 4.5 and 9.0. This result suggests that the pH of bacterial cultures can differentially affect *B. pseudomallei* plc1 gene expression. In contrast, the level of expression of plc3 was similar at all pH values tested. Expression of the plc2 gene was not detected under any of the conditions tested (Fig. 1a) (full-length gels are presented in Supplementary Fig. 1). Our finding that plc2 was not expressed in strain K96243 is similar to the results reported by Ooi et al.\(^\text{18}\). However, plc2 is expressed in *B. pseudomallei* strain 22\(^\text{18}\), and the Plc2 protein was detected in *B. pseudomallei* strain MSHR668\(^\text{18}\) culture supernatant. These findings indicate that plc2 gene expression is strain-dependent.

**The *B. pseudomallei* plc genes are induced in infected macrophages.** We next investigated expression of the plc genes in macrophages. J774A.1 macrophage-like cells were infected with *B. pseudomallei* K96243. At 2, 4, and 6 h post-infection (p.i.), the cells were lysed, and mRNAs corresponding to the plc gene detected using RT-PCR. The results showed no expression of the plc1, plc2 or plc3 genes at 2 h p.i. The expression of all the plc genes was detected at 4 h p.i., but the level of expression of plc2 was relatively higher than the levels of expression of plc1 and plc3. At 6 h p.i., the levels of expression of all plc genes increased compared with 4 h p.i. (Fig. 1b). Our finding that the expression of plc2 gene was induced in macrophages, even though we could not detect expression in culture medium (Fig. 1a), suggests a role of Plc2 in the infection of macrophages.

We stained infected macrophages for LAMP-1 and *B. pseudomallei* using probes labelled with Alexa Fluor 488 or Alexa Fluor 568 and nuclei were stained with DAPI (Fig. 1c). This revealed that at 2 h p.i. most *B. pseudomallei* K96243 were trapped within the phagosome. However, at 4 h p.i., co-localization of *B. pseudomallei* with lysosomes was rare indicating bacterial escape from phagosomes, and almost all of the *B. pseudomallei* cells were not associated with the phagosome at 6 h p.i. It is possible that the intracellular environment induces expression of plc1, plc2 and plc3 genes.

Several previous reports show the expression of plc genes of other species of bacteria in host tissues\(^\text{12,13}\). For example, the *Mycobacterium tuberculosis* plc genes are up-regulated in macrophages, and the Plcs are cytotoxic to mouse macrophages\(^\text{18,19}\). The *Clostridium perfringens* PLC (α-toxin) is produced in host tissues and can activate the arachidonic acid cascade in cells, with consequent modulation of host immune responses\(^\text{20}\), and the induction of ERK1/2 pathway, resulting in cytotoxicity\(^\text{18}\).

**B. pseudomallei plc2, but not plc1 or plc3, is required for bacterial survival and replication in macrophages.** To provide insight into the role of plc1, plc2 and plc3 genes in virulence, we tested a range of single and multiple mutants. We have previously constructed plc1, plc2 and plc12 mutants and these were included in our study. Additionally, for this study we constructed plc3 single and plc123 mutants by insertion mutagenesis\(^\text{14}\). Mutagenesis of the plc genes was confirmed by Southern blotting (data not shown). J774A.1 macrophage-like cells were infected with the mutants. At 2, 4, 6 and 8 h p.i., the numbers of recoverable plc2, plc12 or plc123 mutants were significantly lower (*P < 0.05, **P < 0.01, ***P < 0.001) than the number of viable wild-type bacteria (Fig. 2). In contrast, there was no significant difference (*P > 0.05) in the numbers of viable single plc1 or plc3 mutants compared with the number of viable wild-type bacteria at all tested time points (2, 4, 6 or 8 h p.i.). These findings indicate that plc2 gene was required for *B. pseudomallei* survival and replication inside the macrophage. Our results also indicate that plc1 and plc3 play no role in survival and replication in macrophages, but we cannot discount the possibility of functional redundancy between these enzymes, which would mask the phenotype associated with the single plc1 and plc3 mutants.

To investigate whether the phenotypes we observed with the plc mutants was due to polar effects on down-stream genes we measured expression of the genes downstream of plc1, plc2 or plc3 (bpsl2404, bpsl0337 or bpsl0068 respectively) using RT-PCR. *B. pseudomallei* wild-type and the plc mutants were cultured in LB broth, or extracted from intracellular bacteria after macrophage infection. We demonstrated similar bpsl2404, bpsl0337 and bpsl0068 amplicons with mRNA from wild-type or plc1, plc2, or plc3 mutants cultured in LB broth (Fig. 3a) and extracted from intracellular bacteria (Fig. 3b) (full-length gels are presented in Supplementary Fig. 2).
Figure 1. Reverse transcription (RT)-PCR analysis of *B. pseudomallei* plc expression. (a) Expression of *plc* genes in LB broth adjusted to pH 4.5, 5.0, 7.0, 8.0 or 9. *B. pseudomallei* wild-type K96243 was incubated for 15 min in LB broth at the pH indicated before RNA extraction. Positive control (+ ve) was *B. pseudomallei* genomic DNA. DNase-treated bacterial RNA was used as a negative control (− ve) to confirm the absence of DNA contamination in RNA samples (full-length gels are presented in Supplementary Fig. 1). (b) Expression of *plc* genes in J774A.1 macrophage-like cells infected with *B. pseudomallei*. RNA of *B. pseudomallei* K96243 was harvested from infected macrophages at 2, 4 or 6 h.p.i. and converted to cDNA for PCR analysis with primers specific to each *plc* gene. Positive control (+ ve) was *B. pseudomallei* genomic DNA. DNase-treated bacterial RNA was used as a negative control (− ve). Unfortunately we are not able to reproduce the full length image of this gel because this image file was inadvertently deleted after this project was completed and before we submitted this manuscript. (c) Confocal micrographs of J774A.1 macrophages infected with *B. pseudomallei* (MOI 10) showed that majority of *B. pseudomallei* were within phagosomes at 2 h post-infection (p.i.). Escape of bacteria from the vacuoles was first observed at 4 h p.i., and most bacteria were within the cytosol at 6 h p.i.
These results indicate that the insertional mutation in the \( \text{plc1} \), \( \text{plc2} \), or \( \text{plc3} \) genes did not abolish the expression of downstream genes. However, we cannot discount the possibility that expression of downstream genes was affected.

The defect in intracellular survival of the \( \text{plc2} \) mutant is not due to delayed escape from the phagolysosome. The ability of \( B. \text{pseudomallei} \) to escape from the phagosome thought to be a mechanism by which the bacteria evade phagocyte killing. We had already showed that the \( \text{plc1} \), \( \text{plc2} \) and \( \text{plc3} \) genes are expressed in macrophages (Fig. 1c) and that the \( B. \text{pseudomallei} \) \( \text{plc2} \) mutant was defective in survival in macrophages (Fig. 2). Here we investigated whether Plc2 enzyme played a role in escape from the phagolysosome. \( J774 \) macrophage-like cells were infected with either \( B. \text{pseudomallei} \) wild-type, \( \text{plc2} \), \( \text{plc1} \), \( \text{plc2} \), or \( \text{plc1} \) mutants, and co-localization with lysosomes at 3 h p.i. was investigated by immunostaining with antibodies specific to LAMP-1. A \( B. \text{pseudomallei} \) \( \text{bipB} \) mutant, which is known to be delayed in phagosome escape\(^{22,23} \), was included as a control in our experiments. As expected, we found that the majority of \( \text{bipB} \) mutant cells showed delayed escape from phagosome as evidence the increased association with LAMP-1 (86 ± 3.3% association) when compared with the wild-type bacteria (Fig. 4a). The \( B. \text{pseudomallei} \) \( \text{plc2} \), \( \text{plc1} \) and \( \text{plc2} \) mutants rarely co-localized with LAMP-1 (20.3 ± 2.3%, 20.7 ± 3.5%, and 27.3 ± 3.5% co-localization, respectively; Fig. 4b), similar to the degree of co-localization of the wild-type bacteria with LAMP-1 (17.7 ± 1.5% co-localization). This finding suggests that mutation of the \( \text{plc} \) genes did not affect escape of \( B. \text{pseudomallei} \) from phagosome.

\( B. \text{pseudomallei} \) \( \text{plc2} \) mutant shows deficiency in plaque formation. We next measured plaque formation in monolayers of HeLa cells infected with either \( B. \text{pseudomallei} \) wild-type or the \( \text{plc1} \) \( \text{plc2} \), \( \text{plc3} \), \( \text{plc1} \) or \( \text{plc1} \) mutants. Plaque-formation reflects the ability of bacteria to invade, survive within and then spread from cell to cell. As shown in Fig. 5, plaque-formation in HeLa cells was significantly reduced in cells infected with the \( \text{plc2} \), \( \text{plc1} \) or \( \text{plc1} \) mutants, compared to the wild-type strain (\( **P<0.01 \), \( ***P<0.001 \), \( ****P<0.001 \), respectively). In contrast, there was no significant reduction in plaque formation after infection with the \( \text{plc1} \) or \( \text{plc3} \) mutants (\( P>0.05 \)) (Fig. 5). This result shows correlation with our previous study\(^{14} \) which showed that plaque-formation efficiency in HeLa cells was significantly reduced after infection with \( \text{plc2} \) or \( \text{plc1} \) double mutants compared to the wild-type strain. Plaque-formation was restored in a \( \text{plc2} \) complemented strain. This finding suggested that the defective phenotype was due to the \( \text{plc2} \) gene mutation\(^{14} \) However, complementation of \( \text{plc1} \) double and \( \text{plc1} \) triple mutants was not possible because of restrictions on the use of multiple antibiotic resistance markers in \( B. \text{pseudomallei} \).
To assess whether the reduction in plaque formation reflects a reduced ability to adhere to or to invade HeLa cells, we assessed invasion efficiency. There was no significant difference ($P > 0.05$) in the number of culturable intracellular bacteria at 2 h p.i. between wild-type and either plc2, plc12 double, or plc123 triple mutants (Fig. 6). This finding indicates that the absence of the plc2 gene had no effect on the bacteria to adhere to or invade HeLa cells. Overall, our findings indicate that Plc2 is required for survival and replication of B. pseudomallei in non-phagocytic cells.

The plc12 and plc123 mutants are attenuated in G. mellonella. Our results above showed that Plc2 was required for intracellular survival and replication in host cells. To investigate the roles of Plc1, Plc2 and Plc3 in virulence of B. pseudomallei, a G. mellonella larva infection model was used\(^24\). There was no significant difference ($P > 0.05$) in the survival of larvae infected with the plc1, plc2 or plc3 mutants compared with the wild-type strain (Fig. 7). However, larvae infected with the plc12 or plc123 mutants showed significantly ($P = 0.0031$, $P = 0.0018$, respectively) increased survival, compared to larvae infected with the wild-type strain. This finding suggests redundancy of the functions of the phospholipases in virulence of B. pseudomallei. Our finding that there was no significant difference ($P > 0.05$) in the survival of larvae infected with the plc12 and plc123 mutants suggests that plc3 does not contribute to virulence in G. mellonella larvae. Because of the restrictions on the number of antibiotic markers we could introduce into B. pseudomallei, we could not generate plc12 or plc123 complemented mutants further validate our findings.

Our finding contrasts with a previous study where a plc3 mutant was found to 104-fold attenuated Syrian hamsters compared to the wild-type bacterium\(^16\). This might reflect differences in the immune system between G. mellonella and mammals. G. mellonella possess an innate immune system which involves a cellular immune response mediated by hemocytes, and a humoral immune response orchestrated by antimicrobial peptides\(^25\). However, they lack the complement system found in mammals and G. mellonella also lacks an adaptive immune system\(^26\). Additionally, it is known that B. pseudomallei infection of hamsters is not similar to infection of other mammals such as mice. Hamsters are highly susceptible to infection with B. pseudomallei whereas mice are relatively resistant\(^27\). There are also reports of the different behaviour of B. pseudomallei mutants in hamsters and in mice. For example, fliC\(^28\) and fliD\(^20\) mutants are not attenuated in hamsters but a fliC mutant is highly attenuated in mice\(^29\).

Figure 3. Expression of genes downstream of plc1, plc2 or plc3, assessed using RT-PCR. The mRNA from wild-type, plc1, plc2, plc3, plc12, or plc123 mutants cultured in LB broth (a), or isolated from infected J774A.1 macrophages (b) was extracted before converting to cDNA as outlined in material and methods (full-length gels are presented in Supplementary Fig. 2). The cDNA was amplified using PCR primers specific to the bpsl2404, bpsl0337 or bps0068 genes which are downstream of plc1, plc2 or plc3, respectively (upper panel). The 16S rRNA (middle panel) and DNase-treated mRNA (lower panel) were included as a normalization control and negative control, respectively.
Figure a: Bar graph showing the percentage of Burkholderia-associated LAMP-1 for different mutants. The x-axis represents the different mutants: Wild-type K96243, plc2 mutant, plc12 double mutant, plc123 triple mutant, and bipB mutant. The y-axis represents the percentage of Burkholderia-associated LAMP-1.

Figure b: Images showing the expression of LAMP-1 and DAPI staining in different mutants compared to the wild type. The images are labeled as Wild type K96243, bipB mutant, plc2 mutant, plc12 double mutant, and plc123 triple mutant.
EcoPseudomonas plc3 mutant was selected on (v/v) heat-inactivated fetal bovine serum (Invitrogen) under a 5% CO2 atmosphere at 37 °C in a humidified Culture Collection (ATCC) and were cultured in Dulbecco's Modified Eagle medium supplemented with 10% National Center for Genetic Engineering and Biotechnology (BIOTEC). Gene expression within macrophages, monolayers of J774A.1 murine macrophage-like cells were cultured in each condition using TRIZOL (Invitrogen) according to manufacturer’s instructions. The isolated bacterial RNA was then treated with DNase I (Ambion) to remove any genomic DNA contamination before use. To detect B. pseudomallei genes expression within macrophages, monolayers of J774A.1 murine macrophage-like cells were infected with the bacteria. At the indicated time points, the infected cell monolayers were washed and subsequently lysed with 500 µL of 0.1% Triton X-100 (Sigma-Aldrich) to allow intracellular bacteria released from infected cells. Then, 500 µL of 1 × PBS was added and the intracellular bacterial RNA were extracted using TRIzol (Invitrogen) according to manufacturer’s instructions.

To convert the extracted total RNA to cDNA, SuperScript III First-Strand Synthesis System (Invitrogen) was used. The cDNA was quantified and adjusted so that similar quantities were included in the PCR reactions. The cDNA was amplified using the PCR with primers (Table 1), GoTaq DNA polymerase (Promega) and cycling conditions of 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 s, 50 °C for 15 s and 72 °C for 1 min, and a final extension at 72 °C for 5 min. In each PCR experiment, the amplification of 23S rRNA was used as a normalization control. To construct the B. pseudomallei plc3 mutant, the amplified internal fragment (nucleotide positions 1052–1457) was ligated into EcoRV digested pVK3 plasmid, a suicide vector to generate recombinant plasmid pVSK3 for insertion mutagenesis. The constructed plc3 mutant was selected on Pseudomonas agar base supplemented with SR103E (Oxoid) and chloramphenicol.

To construct the B. pseudomallei plc3 double mutant, the amplified internal plc3 fragment (nucleotide positions 1052–1457) was ligated into EcoRV digested pKNOCK-Cm, a suicide vector to generate recombinant plasmid pVSK3 for insertion mutagenesis. The constructed plc3 mutant was selected on Pseudomonas agar base supplemented with SR103E (Oxoid) and chloramphenicol.

The genes encoding three B. pseudomallei Plc enzymes are expressed within macrophage-like cells, but at different expression levels. The plc2 gene was expressed in infected macrophages but not in culture medium, suggesting a role in virulence. Our findings suggest that plc2 either alone, or in combination with plc1 and plc3, contributes to growth in host cells and our finding that virulence in G. mellonella was dependent on the inactivation of these enzymes, indicates functional redundancy. The data reported in this study provide important new insight into the roles of Plcs in virulence of B. pseudomallei and open new opportunities for further research into the roles on these enzymes in virulence.

Conclusion
The genes encoding three B. pseudomallei Plc enzymes are expressed within macrophage-like cells, but at different expression levels. The plc2 gene was expressed in infected macrophages but not in culture medium, suggesting a role in virulence. Our findings suggest that plc2 either alone, or in combination with plc1 and plc3, contributes to growth in host cells and our finding that virulence in G. mellonella was dependent on the inactivation of these enzymes, indicates functional redundancy. The data reported in this study provide important new insight into the roles of Plcs in virulence of B. pseudomallei and open new opportunities for further research into the roles on these enzymes in virulence.

Materials and methods
Primer, bacterial strains and cell lines. Primers used in this study are shown in Table 1. Escherichia coli, B. pseudomallei K96243 and the mutant strains were routinely cultured in Luria–Bertani (LB) or trypticase soy medium. B. pseudomallei K96243 plc1, plc2 single and plc12 double mutants were constructed in the previous study. All cultures were typically grown for 24–48 h at 37 °C. Appropriate antibiotics (Sigma-Aldrich) i.e. chloramphenicol 50 µg/mL, kanamycin 400 µg/mL and tetracycline 50 µg/mL were added into the medium if required. All manipulations of B. pseudomallei were approved by the Technical Biosafety Committee (TBC), National Center for Genetic Engineering and Biotechnology (BIOTEC).

J774A.1 murine macrophage-like and human epithelial HeLa cells were obtained from the American Type Culture Collection (ATCC) and were cultured in Dulbecco's Modified Eagle medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen) under a 5% CO2 atmosphere at 37 °C in a humidified incubator.

RNA preparation and reverse transcription (RT)-PCR analysis. B. pseudomallei K96243 was grown in LB broth for 6 h before incubation at 37 °C for 15 min in LB broth pH 4.5, 5.0, 6.0, 7.0, 8.0 or 9.0. Total RNA was extracted from 106 CFUs B. pseudomallei cultured in each condition using TRIZOL (Invitrogen) according to manufacturer’s instructions. The isolated bacterial RNA was then treated with DNase I (Ambion) to remove any genomic DNA contamination before use.

To detect B. pseudomallei genes expression within macrophages, monolayers of J774A.1 murine macrophage-like cells were infected with the bacteria. At the indicated time points, the infected cell monolayers were washed and subsequently lysed with 500 µL of 0.1% Triton X-100 (Sigma-Aldrich) to allow intracellular bacteria released from infected cells. Then, 500 µL of 1 × PBS was added and the intracellular bacterial RNA were extracted using TRIzol (Invitrogen) according to manufacturer’s instructions.

To convert the extracted total RNA to cDNA, SuperScript III First-Strand Synthesis System (Invitrogen) was used. The cDNA was quantified and adjusted so that similar quantities were included in the PCR reactions. The cDNA was amplified using the PCR with primers (Table 1), GoTaq DNA polymerase (Promega) and cycling conditions of 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 s, 50 °C for 15 s and 72 °C for 1 min, and a final extension at 72 °C for 5 min. In each PCR experiment, the amplification of 23S rRNA was used as a normalization control.

Construction of B. pseudomallei plc3 single and plc123 triple mutants. A B. pseudomallei plc3 mutant was constructed by insertion mutagenesis. A 406-bp (nucleotide positions 1052–1457) internal region of the plc3 gene was amplified from B. pseudomallei K96243 genomic DNA with primers PLC88 and PLC89 (Table 1). The amplified DNA fragment was ligated into EcoRV digested pKNOCK-Cm, a suicide vector to generate recombinant plasmid pVSK3 for insertion mutagenesis. The constructed plc3 mutant was selected on Pseudomonas agar base supplemented with SR103E (Oxoid) and chloramphenicol.

To construct the B. pseudomallei plc123 triple mutant, the amplified internal plc3 fragment (nucleotide positions 1052–1457) was ligated into EcoRV digested pKNOCK-Km. This constructed plasmid, designated pVSK4, was introduced into B. pseudomallei plc12 double mutant. The mutants were selected on Pseudomonas agar base (Oxoid) supplemented with SR103E (Oxoid) containing chloramphenicol, kanamycin and tetracycline (Sigma-Aldrich). The plc3 mutant was verified by PCR and Southern blotting.
Intracellular survival and plaque assays. Intracellular replication of *B. pseudomallei* in macrophage-like cells was assessed as described previously with some modifications. Briefly, J774A.1 murine macrophage-like cells were seeded at a density of $2.5 \times 10^5$ cells per well of a 24-well tissue culture plate and infected approximately 24 h later with *B. pseudomallei* wild-type K96243 or *plc* mutant at a multiplicity of infection (MOI) of approximately 0.5, for 2 h. Then infected cells were overlaid with DMEM medium (Invitrogen) containing gentamicin 128 μg/mL and spectinomycin 256 μg/mL (Sigma-Aldrich) to kill extracellular bacteria. The infected cell monolayers were subsequently lysed at 2, 4, 6 and 8 h p.i. with 0.1% Triton X-100 (Sigma-Aldrich). The numbers of intracellular bacteria were quantified by serial dilution and plating on tryptic soy agar. Bacterial colony
forming units (CFU) were counted after 36–48 h of incubation at 37 °C. Plaque forming assays were performed as described previously. The plaque-forming efficiency was calculated as the number of plaques/bacterial CFU added per well.

Confocal analysis of bacterial co-localization with LAMP-1. The intracellular localizations of *B. pseudomallei* wild-type K96243 and the *plc* mutants in J774A.1 macrophages cells relative to LAMP-1 containing vesicles were investigated according to previously described. Briefly, Macrophages were infected for 2 h at a multiplicity of infection (MOI) of 2 and incubated at 37 °C, 5% CO₂. At different time points, *B. pseudomallei* infected J774A.1 cells were fixed in 4% paraformaldehyde, the monolayers were permeabilized with 0.5% (v/v)
bacteria co-localized with LAMP-1/total number of intracellular bacteria × 100. For the quantitative analysis of tee (U1-05763-2559).

mutants were adjusted to a concentration of 10^4 CFUs per ml in PBS. A 701N fixed needle syringe (Hamilton, B. pseudomallei

ments. To prepare the bacterial culture for infection, the overnight cultures of

< 0.001).

< 0.05, **P (< 0.01, and ***P (< 0.001).

ANOV A followed by Dunnett's post hoc test within the GraphPad Prism version 7.05 for Windows (www.

pare survival curves and the experiments for comparison between groups were performed using the one-way

L. galleria melonella

larvae was tested as described  previously24 with G. mellonella

Table 1. Oligonucleotide primers used in this study.

| Primers | Oligonucleotide sequences (5′-3′) | Purposes | Sources |
|---------|----------------------------------|----------|---------|
| PLC88 | AGACCCGTCGCTGTCTGTGA | Forward primer for construction of plc3 mutant This study |
| PLC89 | GGCCTGTTGTCGTCGTGA | Reverse primer for construction of plc3 mutant This study |
| Plc1F | TGATGCAGAGAAACCGCTC | Forward primer for internal fragment of plc1 gene This study |
| Plc1R | AGCCGTGACCAGATGACCTAG | Reverse primer for internal fragment of plc1 gene This study |
| Plc2F | GCTCGACAAACAGGCATTACG | Forward primer for internal fragment of plc2 gene This study |
| Plc2R | TTCTGACGATTTGCTGCCC | Reverse primer for internal fragment of plc2 gene This study |
| Plc3F | TCAAGGAAGACATCCGTGCCG | Forward primer for internal fragment of plc3 gene This study |
| Plc3R | CGTGAAATATCCAGAGCAGC | Reverse primer for internal fragment of plc3 gene This study |
| 23s F | TTTCCCAGCTTAGTACGTT | Forward primer for internal fragment of 23s RNA gene *** |
| 23s R | AAGGTTACTCTGGGGAAAA | Reverse primer for internal fragment of 23s RNA gene ** |
| 16s F | AGACACGCGCCAGACTCTTAC | Forward primer for internal fragment of 16s RNA gene ** |
| 16s R | CAGTCAACCGATCGTCCCA | Reverse primer for internal fragment of 16s RNA gene ** |
| bpl2240F-173 | GGCAAGGATCTGCAAAAAAGG | Forward primer for amplification of bpl2240 gene This study |
| bpl2240R-173 | ACGGACCACCTCTTTGTCG | Reverse primer for amplification of bpl2240 gene This study |
| bpl2240R-184 | TCCCGCATCTCTCTCTGATT | Forward primer for amplification of bpl2037 gene This study |
| bpl2037R-184 | ATGCAACACACGGAACAACC | Reverse primer for amplification of bpl2037 gene This study |
| bpl2037R-158 | CTGCCGATGCGCGGATATACA | Forward primer for amplification of bpl0068 gene This study |
| bpl0068R-158 | AAGCAATTCTTGCTGGG | Reverse primer for amplification of bpl0068 gene *** |

Triton X-100, and blocked with 1% (w/v) bovine serum albumin. Bacteria were detected with a 1:10 dilution of mouse anti-Burkholderia monoclonal antibody and detected with a 1:200 dilution of Alexa Fluor 568-goat anti-mouse IgG (Invitrogen, USA). LAMP1 was stained green with a 1:100 dilution of rat monoclonal antibody (1D4B; Abcam, USA) and nuclei were stained blue with a 1:500 dilution of 4,6-diamidino-2-phenylindole (DAPI). Cells were examined by a laser-scanning confocal microscope equipped with LSM 5 Image Browser (LSM 510 META, Carl Zeiss, Germany). The association of Burkholderia with LAMP1 was considered when the red fluorescent bacteria co-localized with the green fluorescence of LAMP1-positive vacuoles, represented as an area of yellow staining.

The percentage of intracellular Burkholderia associated with LAMP-1 was determined as the number of bacteria co-localized with LAMP-1/total number of intracellular bacteria × 100. For the quantitative analysis of the association of intracellular B. pseudomallei strains with LAMP-1 containing vesicles, at least 200 individual bacteria associated LAMP-1 containing vesicles were monitored.

Virulence in G. mellonella. Virulence in G. mellonella larvae was tested as described previously38 with some modifications. Larvae between 2–2.5 cm and free of melanization or injury were used in the experiments. To prepare the bacterial culture for infection, the overnight cultures of B. pseudomallei wild-type and the mutants were adjusted to a concentration of 10^7 CFUs per ml in PBS. A 701N fixed needle syringe (Hamilton, Nevada) was used to inject 10 μl aliquots of the bacterial suspension into the Galleria mellonella larvae to get the final concentration of 10^5 CFUs. Injections were performed directly into the larval body cavity and groups of 10 larvae were injected with each bacterial strain. Control larvae were injected with PBS. Following injection, larvae were incubated in the dark at 37 °C and the number of dead larvae were recorded at a variety of times post injection. The Galleria mellonella study was approved by the Mahidol University-Institute Animal Care and Use Committee (U1-05763-2559).

Statistical analysis. For in vivo mutant characterization, a log-rank (Mantel-Cox) test was used to compare survival curves and the experiments for comparison between groups were performed using the one-way ANOVA followed by Dunnett’s post hoc test within the GraphPad Prism version 7.05 for Windows (www.graphpad.com, GraphPad Software, CA, USA). P-values less than 0.05 were considered statistically significant (*P < 0.05, **P < 0.01, and ***P < 0.001).

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Competing interests
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