Characterization of two distinct phospholipase C enzymes from *Burkholderia pseudomallei*

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*Burkholderia pseudomallei* is a serious bacterial pathogen that can cause a lethal infection in humans known as melioidosis. In this study two of its phospholipase C (PLC) enzymes (Plc-1 and Plc-2) were characterized. Starting with a virulent strain, two single mutants were constructed, each with one *plc* gene inactivated, and one double mutant with both *plc* genes inactivated. The single *plc* mutants exhibited decreased extracellular PLC activity in comparison to the wild-type strain, thereby demonstrating that the two genes encoded functional extracellular PLCs. Growth comparisons between the wild-type and PLC mutants in egg-yolk-supplemented medium indicated that both PLCs contributed to egg-yolk phospholipid utilization. Both PLCs hydrolysed phosphatidylcholine and sphingomyelin but neither was haemolytic for human erythrocytes. Experimental infections of eukaryotic cells demonstrated that Plc-1 itself had no effect on plaque-forming efficiency but it had an additive effect on increasing the efficiency of Plc-2 to form plaques. Only Plc-2 had a significant role in host cell cytotoxicity. In contrast, neither Plc-1 nor Plc-2 appeared to play any role in multinucleated giant cell (MNGC) formation or induction of apoptotic death in the cells studied. These data suggested that PLCs contribute, at least in part, to *B. pseudomallei* virulence and support the view that Plc-1 and Plc-2 are not redundant virulence factors.

INTRODUCTION

Phospholipases are found in a wide variety of Gram-positive and Gram-negative bacteria and are generally categorized by substrate specificity. Those in the phospholipase C class (PLCs) play a significant role in bacterial pathogenesis. Some of these cleave the phospholipid phosphatidylcholine (PC) to produce phosphorylcholine and diacylglycerol (DAG) whilst some cleave other important phospholipid substrates such as phosphatidylinositol (PI) or sphingomyelin (SM). The production of DAG may be particularly important in pathogenesis. DAG itself or its downstream metabolites such as arachidonic acid can contribute to eukaryotic signalling events, including inflammatory processes (Titball, 1998). Moreover, some PLCs, including the *Clostridium perfringens* z-toxin and the haemolytic PLC (PlcH) of *Pseudomonas aeruginosa*, also hydrolyse SM to generate ceramide. This can induce apoptosis in eukaryotic cells (Stonehouse *et al.*, 2002; Titball, 1993). Although bacterial PLCs are thought to be key virulence factors in several infectious diseases, the pathogenic mechanisms are quite varied. For example, clostridial z-toxin is a PLC with haemolytic and lethal dermonecrotic and platelet-aggregating properties (Jepson & Titball, 2000) whilst that of *Listeria monocytogenes* functions to allow the organism to escape from intracellular phagolysosomes (Grundling *et al.*, 2003; Smith *et al.*, 1995). Purified PlcH preparations from *P. aeruginosa* are cytoxic since injection into mice causes hepatonecrosis and renal tubular necrosis (Meyers *et al.*, 1992). This PlcH also inhibits the bacterium-induced neutrophil respiratory burst by interfering with a protein kinase C-dependent, non-p38 kinase-dependent pathway (Terada *et al.*, 1999).

*Burkholderia pseudomallei* is a pathogen that can cause a severe infection in humans called melioidosis. One of the possible disease outcomes is a fulminant septicaemia which if left untreated is rapidly fatal. The organism is acquired...
through disrupted skin or by inhalation from environmental sources, where it resides as a saprophyte (White, 2003). It produces several potential virulence factors including catalase, peroxidase, superoxide dismutase, haemolysin, lipase and PLC (Woods et al., 1999). Production of PC-hydrolysing PLC (PC-PLC) can be detected as a zone of opalescence surrounding colonies grown on agar supplemented with egg-yolk emulsion. We first cloned and characterized the gene encoding PLC from Burkholderia thailandensis (formerly known as B. pseudomallei biotype Ara†). This 73 kDa Plc-1 protein (accession no. AF107252) shares significant similarity (more than 40 %) with both the haemolytic PLC (PlcH) and the non-haemolytic PLC (PlcN) from P. aeruginosa (Korbsrisate et al., 1999). In 2002, we sequenced another B. thailandensis plc gene, plc-2, and deposited the sequence in the GenBank database (AY114143). However, these PLCs have not been investigated further at the level of detail reported here for the B. pseudomallei PLCs.

In this study we characterized two distinct plc genes identified through a search of the published B. pseudomallei K96243 genome sequence (Holden et al., 2004). We also examined the functions of their products (Plc-1 and Plc-2) in challenged eukaryotic cells by constructing three B. pseudomallei plc mutants and comparing these with the wild-type strain. The assays carried out tested the ability to survive in phospholipid-rich culture medium, and to induce cytotoxicity, apoptosis and the formation of multinucleated giant cell (MNGC) in macrophage cell lines.

**METHODS**

**Strains, plasmids, media and growth conditions.** Table 1 lists the bacterial strains and plasmids used in this study. All B. pseudomallei and Escherichia coli strains were routinely maintained in Luria–Bertani (LB) or trypticase soy medium. Pseudomonas agar base supplemented with SR103E (cetrimide, fucidin and cephalosporin) from Oxoid was used after conjugation as a selective medium to inhibit growth of E. coli. All cultures were grown at 37 °C. Tetracycline (Tc, 60 μg ml⁻¹), chloramphenicol (Cm, 40 μg ml⁻¹) and ampicillin (Ap, 100 μg ml⁻¹) were added to media when required.

Murine macrophage (RAW264.7) and human epithelium (HeLa) cell lines were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco’s modified Eagle’s medium (Gibco-BRL) supplemented with 10% fetal bovine serum (HyClone) at 37 °C under a 5 % CO₂ atmosphere.

**Cloning of plc-1 and plc-2.** B. pseudomallei plc-1 and plc-2 ORFs were amplified from chromosomal DNA using the primers PLC-51 (5’-AGCGGATCCATCGAATCATGACGAATCAGAATCGCC-3’) and PLC-49 (5’-TGCAGAATTCGGATCATGTCCATTGCCTG-3’). ORF amplification products were treated with exonuclease I and shrimp alkaline phosphatase, before being inserted into suitable vectors as described below.

**Table 1. Plasmids and strains used in this study**

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<th>Plasmid/strain</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
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<tr>
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<td>Expression vector for E. coli</td>
<td>Invitrogen</td>
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<td>pUC18</td>
<td>Expression vector for E. coli</td>
<td>Gibco-BRL</td>
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<td>pTrcHisA-plc1</td>
<td>B. pseudomallei plc-1 in pTrcHisA</td>
<td>This study</td>
</tr>
<tr>
<td>pUC18-plc1</td>
<td>B. pseudomallei plc-1 in pUC18</td>
<td>This study</td>
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<td>pUC18-plc2</td>
<td>B. pseudomallei plc-2 in pUC18</td>
<td>This study</td>
</tr>
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<td>pKNOCK-Cm</td>
<td>Broad-host-range mobilizable suicide vector</td>
<td>Alexeyev (1999)</td>
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<tr>
<td>pSS-2</td>
<td>2600 bp plc-2 gene from B. pseudomallei in pKSII(−) (Stratagene)</td>
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<td>pSSK-2</td>
<td>pKNOCK-Tc carrying 350 bp PstI–NorI fragment from pSS-2</td>
<td>This study</td>
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<td>pBBR-plc2</td>
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<td>This study</td>
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<td>Wild-type strain</td>
<td>Clinical isolate</td>
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<td>JSK-4</td>
<td>B. pseudomallei plc-1 knockout mutant (plc-1 : : pJSK-4); produces extracellular PC-PLC</td>
<td>This study</td>
</tr>
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<td>SSK-2</td>
<td>B. pseudomallei plc-2 knockout mutant (plc-2 : : pSSK-2); produces extracellular PC-PLC</td>
<td>This study</td>
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<tr>
<td>SSK-12</td>
<td>B. pseudomallei plc-1 plc-2 double knockout mutant (plc-1 : : pJSK-4, plc-2 : : pSSK-2)</td>
<td>This study</td>
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<td>SSK-2-plc2</td>
<td>B. pseudomallei SSK-12 carrying plasmid pBBR-plc2</td>
<td>This study</td>
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<td>E. coli S17-1 Δpir</td>
<td>Tp³ Sm³ recA, thi, pro, hsdR M⁺ RP4 : 2-Tc : Mu : Km Tn7zipir</td>
<td>de Lorenzo &amp; Timmis (1994)</td>
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</table>
B. pseudomallei Plc-1 and Plc-2

(B'GTGATCATGACTGAGCCGCGC-3')/PLC-50 (5'-GGGAT-ATTACCTCGTATCCGTCGCTTGC-3'), respectively. Underlined sections of each primer indicate restriction sites, BamHI and EcoRI. Recombinant DNA techniques were carried out by standard procedures. PCR products were cloned into pTrcHisA (Invitrogen) to create pTrcHisA-plc1 and pTrcHisA-plc2. The recombinant plasmids were transferred to E. coli DH5α (Stratagene). Nucleotide sequences of DNA inserted onto both plasmids were determined by using an ABI Prism BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems). To confirm enzymic activity, both plc-1 and plc-2 ORFs were also cloned into plasmid pUC18 (Table 1).

**Enzymic and biological activities.** PC-PLC activity was measured (Kurioka & Matsuda, 1976) by hydrolysis of p-nitrophenolphosphorylcholine (pNPPC; Sigma) measured using a spectrophotometer at 410 nm. This value was divided by the optical density of bacterial cells at 590 nm to give the relative enzymic activity normalized to cell number. PC-PLC and sphingomyelinase (SMase) activity were also measured, as described previously (van Echten-Deckert, 2000) using BODIPY-labelled PC or NBD-labelled SM. Mixed-micelle assays were performed with BODIPY-labelled PC and SM (Molecular Probes). A typical assay consisted of adding 5 μl Plc-1 or Plc-2 sample to 2 μl (0.2 μg) NBD SM in 25 mM HEPES (pH 7.2), 100 mM NaCl and 0.4 mM octyl β-D-glucopyranoside. The samples were incubated at 37 °C for 0, 30, 60 or 90 min, at which time SMase activity was assessed by measuring the release via TLC using a H2O-saturated ethyl acetate/iso-octane/glacial acetic acid (135: 75: 30, by vol.) solvent system. BODIPY- and NBD-labelled products were detected using a Bio-Rad Fluor-S MultiImager. Image analysis was carried out using Quantity One version 4.0.3 (Bio-Rad). PLCs can be classified as haemolytic or non-haemolytic depending on their ability to cause erythrocyte lysis. The haemolytic activity of recombinant Plc-1 and Plc-2 was tested as described by Ostroff et al. (1990) using human erythrocytes.

**Construction of plc-1, plc-2 and double plc-1 plc-2 knockout mutants.** The B. pseudomallei plc-1 knockout mutant was created by inserting a Cm resistance plasmid into the plc-1 gene on the bacterial chromosome as described previously (Korbsrisate et al., 2005; Loprasert et al., 2002). In short, a 500 bp internal fragment of B. pseudomallei plc-1 was amplified by PCR using primers PLC-F2 (5'-GGATATTACCTCGTATCCGTCGCTTGC-3') and PLC-B2 (5'-GATGAATTGC-9), and the product was cloned into suicide vector pBBR1MCS (Kovach et al., 1995) to give pBBR-plc2. The recombinant plasmid was transferred from E. coli K96243 to B. pseudomallei SSK-2 by conjugation and selected on Pseudomonas agar supplemented with Cm. The resulting B. pseudomallei SSK-2-plc2 strain was subjected to plasmid isolation to confirm the presence of plasmid pBBR-plc2 in the bacterial cells.

**Multiplication of plc mutants in egg-yolk medium.** The plc mutants and B. pseudomallei K96243 were separately cultured in sterile deionized water at room temperature (1 and 6 weeks) to generate bacterial starvation conditions. At indicated times the B. pseudomallei cultured in deionized water were aspirated into M9 minimal culture broth (Vasil et al., 1990) containing an egg-yolk solution (10%, v/v). The numbers of bacteria in the M9-egg-yolk broth before and after overnight culture at 37 °C were quantified by colony counts and compared. Egg-yolk solution was prepared by diluting freshly obtained egg-yolk with normal saline solution and filtering with a Millex HA filter (pore size, 0.45 μm; Millipore).

**Plaque and MNGC formation assays.** Burkholderia-induced plaque assays (Kespichayawattana et al., 2000) were performed as described earlier. Essentially, plaque assays were done by infecting HeLa cells with B. pseudomallei at a m.o.i. of approximately two bacteria per cell and overlaid with an agarose medium containing kanamycin (Km, 250 μg ml⁻¹). To enhance visualization, plaques were overlaid with agarose containing an additional 0.01% neutral red and observed 4 h later. Plaque-forming efficiency was determined by taking the number of plaques and dividing this by the bacterial c.f.u. added per well.

To investigate the potential role of Plc in MNGC formation, B. pseudomallei was infected J774A.1 murine macrophage-like cells and stained as described previously (Kespichayawattana et al., 2000). The percentage of MNGC formation was determined by dividing the number of nuclei within MNGC by the total number of nuclei counted and multiplying the result by 100.

**Cytotoxicity and apoptosis detection.** The cytotoxic ability of the plc mutants was determined by incubating a RAW264.7 macrophage cell line with either wild-type or plc knockout mutants at a m.o.i. of approximately two bacteria per cell. After 1 h incubation at 37 °C with 5% CO₂, the cells were washed with pre-warmed PBS. Culture medium containing Km was added and the cell culture was further incubated for 2 h to completely eliminate residual extracellular bacteria. The medium was then replaced with medium containing Km (20 μg ml⁻¹) and incubated for a further 8 h, after which culture supernatants and cell lysates were collected for analysis. Cytotoxicity was quantified colorimetrically with a CytoTox96 lactate dehydrogenase (LDH)-release kit (Promega). The percentage of cytotoxicity was calculated using the formula [(experimental release−spontaneous release)/(total release−spontaneous release)]× 100, in which spontaneous release is the amount of LDH activity in the supernatant of uninfected cells and total release is the activity in macrophage lysates.

Apoptosis detection was performed as previously described (Suparak et al., 2005). Briefly, J774A.1 cells were infected with B. pseudomallei strains at a m.o.i. of approximately 10 bacteria per cell. At 6 h after infection, the supernatant and cells were collected to quantify apoptosis levels using an annexin V-FITC detection kit (BD Biosciences) according to the manufacturer’s instructions.

**Statistical analysis.** All tests for significance were performed using the Student t-test in Excel software 97 (Microsoft). Results were considered significant at a P value of <0.05.
RESULTS AND DISCUSSION

Comparison of primary structure and enzymic activity

BLASTP search of B. thailandensis plc-1 (AF107252) and plc-2 (AY114143) with the published B. pseudomallei K96243 genome sequence (Holden et al., 2004) indicated the presence of two plc genes on chromosome 1 and another on chromosome 2. Genes encoding Plc-1 (BPSL 2403) and Plc-2 (BPSL 0338) on chromosome 1 were 2100 and 2115 nucleotides in length, respectively. The predicted molecular masses of Plc-1 and Plc-2 were both 77 kDa, whilst that of the third PLC (plc-3, BPSS 0067) encoded on chromosome 2 was 81 kDa. Both Plc-1 and Plc-2 were predicted to be acidic proteins (pl 6.03 and 6.24 respectively); this acidity was similar to that of PlcH in P. aeruginosa (pl 5.96). The third PLC (on chromosome 2) was predicted to be a basic protein (pl 8.16), a characteristic shared with PlcN (pl 8.8) in P. aeruginosa. Plc-1 and Plc-2 are 51% identical and 61% similar to each other. Plc-1 and Plc-2 are both ~43% identical and 54% similar to Plc-3. Plc-1 and Plc-2 are ~47% identical and ~60% similar to the PlcN of P. aeruginosa and both are ~42% identical and 54% similar to the PlcH of that organism. Most notably, the sequence of Plc-1 is nearly identical (a single amino acid difference: arginine changed to glutamine) to one (BMA0584) of the two Burkholderia mallei (Nierman et al., 2004) predicted PLCs, and the B. pseudomallei Plc-3 is completely identical to the other PLC encoded in the B. mallei genome (BMAA 0079). These data would suggest that B. mallei has lost, or never acquired, the plc-2 gene but has retained plc-1 and plc-3 of B. pseudomallei.

A comparison of the molecular characteristics of B. pseudomallei Plc-1 with B. pseudomallei Plc-2, B. thailandensis PlcN1 and PLCs from P. aeruginosa revealed several similar properties (Table 2). All contained 34–38 amino acid signal peptide sequences, which are unusual in their length and in the presence of phenylalanine and charged residues (Korbsrisate et al., 1999; von Heijne, 1985). A number of features in the putative signal sequences are similar to those of both P. aeruginosa PLCs that are known to be secreted through the inner membrane via the twin arginine translocase (TAT) pathway (Ochsner et al., 2002; Voulhoux et al., 2001). These features of the B. pseudomallei PLCs and the fact that this pathogen, as well as B. mallei, carry homologues of the genes encoding TatABC proteins suggest that B. pseudomallei PLCs are likewise secreted via the TAT pathway rather than the Sec pathway. The leader sequences of all three B. pseudomallei PLC homologues (and the two homologues in B. mallei) have twin arginine leader sequences. More recently, we have found that the B. pseudomallei tatABC operon can complement a ΔtatABC mutant of P. aeruginosa, restoring PlcH secretion into the culture supernatant (A. P. Tomaras & M. L. Vasil, unpublished data).

Analysis of the substrate-degrading capability of E. coli carrying pUC18 : : plc1 or pUC18 : : plc2 indicated that both Plc-1 and Plc-2 are able to hydrolyse PC and SM (Fig. 1). Plc1 was more active on PC (Fig. 1a, b) and SM (Fig. 1c) than Plc2, but at this time this is based on a semiquantitative assessment. Periplasmic extracts of E. coli carrying only pUC18 did not demonstrate any detectable production of ceramide from SM over the 2 h period of the assays. A more complete enzymic analysis awaits purification of each of these enzymes. However, neither exhibited any significant haemolytic activity on human erythrocytes (data not shown). While PlcH is haemolytic and capable of attacking both PC and SM, other PLCs (e.g. PC-PLC of L. monocytogenes) can hydrolyse these substrates are not known to be haemolytic. Whether Plc-1 or Plc-2 can act alone on phospholipids packed in a membrane will have to be assessed in additional experiments.

The fact that B. pseudomallei encodes at least three PLC enzymes (Holden et al., 2004) raises questions about possible functional redundancy. As noted earlier, other organisms, such as L. monocytogenes (Mengaud et al., 1991; Ravenneau et al., 1992) and Mycobacterium tuberculosis (Raynaud et al., 2002), carry up to four genes encoding PLCs. It is likely that these PLC enzymes have different roles or act at different stages of host infection as do PLCs reported for other bacteria (Mengaud et al., 1991; Ravenneau et al., 1992; Raynaud et al., 2002). Perhaps the

<table>
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<th>PLC enzyme</th>
<th>Size of predicted mature polypeptide (aa)</th>
<th>pI</th>
<th>Signal peptide (aa)</th>
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<td>35</td>
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<tr>
<td>B. pseudomallei, Plc-1</td>
<td>700</td>
<td>6.03</td>
<td>34</td>
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PLC-encoding genes in *B. pseudomallei* are expressed under different conditions like the genes encoding PlcH and PlcN in *P. aeruginosa*.

**Mutant constructions and effect of plc mutation on multiplication in culture medium**

*B. pseudomallei* plc mutants were constructed and verified (Fig. 2). Determination of PC-PLC activity in *B. pseudomallei* JSK-4 (*plc*-1::pJSK-4), SSK-2 (*plc*-2::pSSK-2) and SSK-12 (*plc*-1::pJSK-4 *plc*-2::pSSK-2) mutants showed that PC-PLC activity still could be detected from the supernatant culture of the three mutants but at a lower level than for the wild-type strain (Table 3). The amount of PC-PLC activity was comparable in JSK-4 and SSK-2 and lowest in SSK-12. Our observations indicated that the two genes encode functional extracellular PLCs.

Despite the well-known role of PLCs in the virulence of both intracellular (e.g. *L. monocytogenes*) and extracellular (*P. aeruginosa* and *C. perfringens*) bacterial pathogens, their involvement in the virulence of *B. pseudomallei* has not been well investigated. This involvement is likely to be multifactorial. One of the possible functions is nutrient acquisition. Using starvation assays followed by subculture in M9-egg-yolk broth (enriched source of PC) we demonstrated that single knockouts of either *plc*-1 or *plc*-2 on either JSK-4 or SSK-2 background were less affected in growth in comparison to the wild-type strain.
Table 3. PC-PLC activity and growth of *B. pseudomallei* wild-type and plc mutants in egg-yolk-supplemented M9 (EY) medium

Results are representative of experiments performed in triplicate using fresh samples each time.

<table>
<thead>
<tr>
<th><em>B. pseudomallei</em></th>
<th>PC-PLC activity* (AA110/OD590)</th>
<th>Relative multiplication in EY medium (%)† after maintaining in distilled water for</th>
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<tr>
<td></td>
<td></td>
<td>1 week</td>
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<tr>
<td>K96243</td>
<td>0.90 ± 0.07</td>
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<td>JSK-4</td>
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<tr>
<td>SSK-12</td>
<td>0.41 ± 0.08</td>
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</table>

*Means±SD of three independent experiments are shown.
†(Increase in c.f.u. after culturing in EY medium/increase in c.f.u. of wild-type after culturing in EY medium) × 100.

*plc-2* (i.e. JSK-4 or SSK-2) could still utilize egg-yolk (Table 3). A possible explanation is that either enzyme can carry out phospholipid hydrolysis or that the knockouts are compensated for by other *plc* genes present in the *B. pseudomallei* genome. In contrast, multiplication of the double *plc-1 plc-2* mutant (SSK-12) after starvation in distilled water was significantly lower than that of the wild-type strain (*P*<0.001), indicating that the PLCs were important in acquisition of nutrients (e.g. phospholipids) under these conditions. However, the fact that the double *plc-1 plc-2* knockout mutant was able to replicate and survive suggested that other PLC enzymes such as the one encoded on chromosome 2 could contribute to hydrolysis of PC for survival.

*B. pseudomallei plc-1 and plc-2 mutations affect plaque formation*

*B. pseudomallei* can spread directly from cell to cell, forming plaques in both phagocytic and nonphagocytic cells (Kespichayawattana *et al.*, 2000). Cellular damage as detected by plaque assays is an important feature of *B. pseudomallei* pathogenesis. The ability of the wild-type and the plc mutants to disseminate from cell to cell intracellularly was evaluated and it was found that plaque-formation efficiency with infected HeLa cells was significantly reduced for the mutants SSK-2 and SSK-12 when compared to the wild-type strain (*P*<0.001 and <0.001, respectively) whereas that for JSK-4 was not (Fig. 3a). The results indicated that Plc-2 plays a significant role in the process. We found that a plasmid-borne *plc-2* gene could restore the defective plaque formation in SSK-2 (Fig. 3a). Removal of Plc-1 alone had no effect on plaque formation, but the plaque-formation efficiency of SSK-12 was significantly lower than that of SSK-2 (*P*=0.0186); this suggested that Plc-1 could have an additive effect to Plc-2. Finally, no apparent difference in growth rate amongst the three mutants was noted (data not shown). This excluded the possibility that differences in growth rate interfered with the observed results.

*B. pseudomallei requires Plc-2 for cell cytotoxicity*

Monitoring the integrity of the cell membrane can be an indication of the level of host cell death taking place. This can be measured as cytoplasmic enzyme activity released by damaged cells. For example, LDH is a stable cytoplasmic enzyme present in all cells and it is rapidly released into the cell culture supernatant upon damage to the plasma membrane. The mutants SSK-2 (plc-2 mutant) and SSK-12 (double *plc-1 plc-2* mutant) but not JSK-4 (*plc-1* mutant) were significantly less cytotoxic (*P*<0.001 and 0.009, respectively) towards RAW264.7 cells than the wild-type strain (Fig. 3b). This suggested that Plc-2, but not Plc-1, contributes to cytotoxicity (i.e. release of LDH). The defect in cell cytotoxicity of mutant SSK-2 was restored in a complementation assay using *B. pseudomallei* strain SSK-2-*plc2* (Fig. 3b), indicating that the phenotypes observed were not due to potential downstream polar effects.

The finding that the LDH levels for cells infected with SSK-2 and SSK-12 were higher than background suggested that other bacterial (e.g. Plc-3) or host cell factors (e.g. cytokine release) may also have been involved in the cytotoxic process of *B. pseudomallei* infection. Recently, Tuanyok *et al.* (2006) reported the importance of *B. pseudomallei* plc-3 (BPSS 0067) as a potential virulence factor in the hamster model of acute melioidosis. The inability of Plc-1 to generate LDH release from infected cells provides evidence that the activities of the two PLC enzymes are not redundant. Plc-2 seems to make the most significant contribution to the virulence phenotypes examined in this study. The detailed mechanism of cytotoxicity is unknown but it is possibly due to the enzymic disruption of target cell membranes as demonstrated with PLCs from C.
perfringens (Krug & Kent, 1984) as well as with other Gram-negative and Gram-positive bacteria (Titball, 1993). The role of cell cytotoxicity in the pathogenesis of B. pseudomallei is unclear. Disruption of host cell membranes might facilitate its entry into the bloodstream and systemic spread. Furthermore, macrophage lysis in vivo might also increase inflammation and the release of enzymes contributing to local tissue destruction. Taking these results together, it is possible that PLC is a virulence factor recognized by the host immune response. This notion is supported by a previous report that melioidosis patients have antibody against B. thailandensis PLC (Korbsrisate et al., 1999).

B. pseudomallei plc-1 and plc-2 have no effect on MNGC formation and induction of apoptosis

The presence of MNGCs has been observed in the tissues of patients with melioidosis (Wong et al., 1995) and we have previously shown that mutation of B. pseudomallei type III translocator protein BipB can reduce MNGC formation (Suparak et al., 2005). In contrast, none of the three plc mutants were defective in their ability to induce MNGC formation (Fig. 3c) under the test conditions used. Upon infection of a macrophage cell line with B. pseudomallei, subsequent growth in the cytoplasm and MNGC formation are dependent on ability of the bacterium to escape from a vacuole. Normal MNGC formation in a double plc-1 plc-2 mutant suggests that Plc-1 and Plc-2 do not play a role in the process. In contrast, the capacity of L. monocytogenes to spread efficiently from cell to cell appears to be dependent on the product of plcB, a secreted PLC with broad substrate specificity including activity on PC and SM (Geoffroy et al., 1991) Transmission electron microscopy revealed that plcB-deficient mutant strains accumulated in double-membrane vesicles in newly infected cells (Vazquez-Boland et al., 1992).

Since B. pseudomallei can induce apoptotic death in infected macrophages (Kespichayawattana et al., 2000) and haemolytic PLC (PlcH) of P. aeruginosa can induce programmed cell death (i.e. apoptosis) in eukaryotic cells (Stonehouse et al., 2002; Titball, 1993), the ability of the wild-type and PLC mutants to induce apoptosis was evaluated. No significant difference in apoptosis induction was observed among the three B. pseudomallei plc mutants (Fig. 3c), suggesting that Plc-1 and Plc-2 do not play a role in this process.

Concluding remarks

We have characterized two B. pseudomallei plc genes located on chromosome 1 that encode functional enzymes able to hydrolyse PC and SM. Analysis of plc mutant behaviour in model eukaryotic cell infections demonstrated that Plc-1, together with Plc-2, contributes to plaque formation. However, only Plc-2 has a significant effect in the cytotoxicity assay used in this study. In contrast, neither Plc-1 nor Plc-2 appeared to play any role in MNGC formation or induction of apoptotic death in this cell model. The data support the view that Plc-1 and Plc-2 are not redundant virulence factors in the survival and pathogenesis of B. pseudomallei but demonstrate that...
Plc-2 makes the most significant contribution to the virulence phenotypes examined in this study.

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