PRODUCTION AND PURIFICATION OF *BURKHOLDERIA PSEUDOMALLEI* BIPD PROTEIN

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Abstract. Type III secretion system (TTSS) clusters contain virulent genes of both animal and plant Gram-negative bacteria. The full length (933 bp) gene of *bipD*, a member of TTSS cluster of *Burkholderia pseudomallei,* was isolated by PCR amplification from *B. pseudomallei* DNA and cloned into pGEX 4T-1. GST-BipD fusion protein and BipD protein obtained by removal of GST using thrombin were employed to detect the presence of *B. pseudomallei* antibodies in sera obtained from melioidosis and non-melioidosis patients by immunoblotting. Sensitivity and specificity of BipD protein was 100% and 91.1%, respectively, whereas that of fusion protein was 77.8% and 90.0%, respectively.

INTRODUCTION

Melioidosis is an infectious disease of humans and animals caused by a soil saprophyte Gram-negative bacterium, *Burkholderia pseudomallei*. Endemic areas of melioidosis are Southeast Asia and northern Australia. The number of melioidosis patients has steadily increased during the past 10 years and melioidosis can be classified as an emerging disease (Dance, 2000). Melioidosis has the following unique features: a wide range of clinical symptoms ranging from asymptomatic, localized infection to a fulminant septicemic pneumonia with high mortality rate; classification as a biological risk category B agent due to its high fatal respiratory infectivity route that could be used as a potential agent for biological warfare; requirement of long term treatment with appropriate drugs and high relapse rate; long dormant state of up to 62 years (Ngauy *et al*, 2005).

Although several virulent factors of *B. pseudomallei* have been reported by *in vitro* experiments, the virulent property of *B. pseudomallei* that has a high possibility to respond to the long dormancy state is the type III secretion system (TTSS) which may facilitate the organism to survive and replicate in nonphagocytic cell (Brett and Words, 2000; Stevens *et al*, 2002). TTSS is a cluster of genes encoding a series of proteins that are delivered as effector proteins from the bacterial cytoplasm into the host cell interior (Blocker *et al*, 2003). The main function of effector proteins is to facilitate entering into and survival in phagosome (He *et al*, 2004). There are three TTSS clusters in *B. pseudomallei.* TTSS1 and TTSS2 are homologous to TTSS of the plant pathogen *Ralstonia solanacearum* (Attree and Attree, 2001; Rainbow *et al*, 2002), while TTSS3 is similar to Inv/Mxi-Spa type III secretion systems of *Salmonella* and *Shigella* (Attree and Attree, 2001; Stevens *et al*, 2002). Recent reports have indicated that TTSS3 is required for virulence of *B. pseudomallei* in an animal model (Stevens *et al*, 2004; Warawa and Woods, 2005).

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Detection of antibodies produced by the host against an antigenic bacterial protein constitutively expressed by a pathogen can be used to identify and classify bacterial infections in patients. A recent report has shown that at least six gene products of *B. pseudomallei* specifically react with pooled absorbed melioidosis serum (Jitsurong *et al,* 2003), one of which is BipD, a protein product of TTSS3 (Stevens *et al,* 2002). Mutation in *bipD* has been shown to disrupt the ability of *B. pseudomallei* to induce membrane protrusion and actin tail and to impair invasion of epithelial cells *in vitro* (Stevens *et al*, 2002, 2004). BipD is required for full virulence in murine model of melioidosis (Stevens and Galyov, 2004) and BipD protein is proposed as being a key component of the translocation apparatus in this bacterium.

In this study, *bipD* was cloned, sequenced and expressed.

MATERIALS AND METHODS

Serum samples

Serum samples were donated from Songklanagarind Hospital, Songkhla, Thailand during January to June 2005. Twenty-seven melioidosis serum samples were obtained from patients who had been confirmed positive for *B. pseudomallei* by culture method, from active infected patient who had *B. pseudomallei* in blood and 4 patients with localized infection. Control sera were obtained from hospitalized patients infected with other bacterial infections: 37 with gram-negative bacilli, 11 gram-positive cocci, and 7 *Mycobacterium tuberculosis*. Fifteen serum samples were also obatined from non-infected patients: 10 with SLE, 3 myocardial infarction (MI) and 2 rheumatoid arthritis. Normal sera were collected from 25 healthy blood donors. All sera were stored at -70ºC until use.

Serum absorption

Pooled sera from melioidosis and non-meliodosis patients were absorbed with a lysate of *E. coli* strain BL21 (Sambrook *et al*, 1989). Pooled sera were diluted 1:2,000 with TBST (25 mM Tris, 0.15 M NaCl, 2.5 mM KCl, 0.05% Tween 20), sodium azide was added at 0.02% final concentration and sera were kept at 4ºC.

Cloning and expression of *bipD*

B. pseudomallei isolated from a melioidosis patient at Songklanagarin Hospital was grown in LB broth overnight at 37ºC. DNA was extracted. The *bipD* was amplified by PCR. Primers used were as follows: forward primer 5′-CGGATCCATGAACATGCATGTCG-3′ and reverse primer 5′-CTGCAGATCTGAG CGGCCGC-3′. *BamH*I and *Not*I site is underlined in the forward and reverse primer, respectively. PCR volume of 25 µl contained 200 ng DNA, 0.04 mM dNTP, PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$ and 0.1% Triton X-100), 2.5 mM $MgCl_2$, 62.5 pmol of primers, 1 unit of *Taq* polymerase (Promega, USA). PCR was performed for 30 cycles as follows: 95ºC for 15 seconds, 45ºC for 30 seconds, 74ºC for 90 seconds. Cycling was initiated by 5 minutes of denaturation at 95ºC and terminated by 12 minutes of incubation at 72ºC. The obtained PCR fragments were cloned into pGEM-T Easy (Promega, USA) and sent for sequencing (Macrogen, South Korea) using SP6 and T7 primers. Then, PCR product containing the *BamH*I and *Not*I site as designed in forward primer and reverse primer, respectively, was cloned into *BamH*I and *Not*I sites of pGEX 4T-1 (GE Healthcare, Sweden).

To express the GST (glutathione S-transferase)-BipD fusion protein, the recombinant plasmid, GST-BipD pGEX 4T-1was transfected into *E. coli* strain BL21 and transformants selected on LB agar containing 100 µg/ml ampicilin for 16-18 hours at 37ºC. A transformant clone was chosen and cultured in 2x YT medium (10 ml) containing 100 µg/ml ampicilin for 16-18 hours at 37ºC. For expression of the recombinant protein, the culture was

added to 100 ml of 2x YT medium containing 100 µg/ml ampicilin and incubated for 1.5 hours at 37ºC. Fusion protein expression was induced with 1.0 mM IPTG (Sigma) and the culture grown for another 3-5 hours. Cells were harvested by centrifugation at 2,874*g* for 20 minutes and the pellet was resuspended in 20 mM Tris HCl, 5 mM EDTA, pH 7.4. The cells were broken with sonication 6 times for 10 seconds and centrifuged at 9,500*g* for 20 minutes. The pellet was suspended with TBS (25 mM Tris, 0.15 M NaCl, 2.5 mM KCl) buffer pH 7.4 and stored at -20ºC.

Purification of fusion protein and BipD protein

The crude fusion protein solution was centrifuged at 1,616*g* for 10 minutes. An aliquot of 1 ml of supernatant was incubated with 1 ml of Glutathione Sepharose 4 Fast Flow beads (GE Healthcare, Sweden) for 1 hour at 4ºC. The beads were then centrifuged at 180*g* for 10 minutes at 4ºC and the supernatant was discarded. Beads were washed 5 times with 12 ml of cold TBS buffer. The fusion protein was eluted with 3 aliquots of 1 ml of elution buffer (50 mM Tris-HCl, 20 mM reduced glutathione pH 8.0).

To prepare BipD protein, GST-tagged protein on the beads after unbound proteins were washed off, was incubated with 0.5 ml of TBS containing 50 units of thrombin solution (Sigma) at 25ºC for 16 hours. Beads were centrifuged at 180*g* for 10 minutes and the supernatant was collected and stored at -20ºC. Protein concentration was determined by Lowry assay (Lowry *et al,* 1951) using bovine serum albumin as standard.

Immunoblotting

Either crude protein (0.6 µg), purified GST-BipD protein or BipD protein (0.04 µg) was separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane (BioTrace®NT). The membrane was incubated with 5% (w/v) skim milk powder for 1 hour, washed 3 times with TBST (25 mM Tris, 0.15 M NaCl, 2.5 mM KCl, 0.05% Tween 20) and incubated with individual human serum, pooled absorbed non-melioidosis serum (as negative control) and pooled absorbed melioidosis serum (as positive control) at a dilution of 1:1,000 in TBST for 1 hour at 25ºC. After washing 3 times with TBST, the membrane was incubated with a secondary antibody (anti-human IgG conjugated with alkaline phosphatase, Promega, USA) at 1:50,000 dilution for 1 hour at 25ºC. After washing 3 times with TBST, secondary antibody was detected with a solution of 0.05 mM nitro blue tetrazolium chloride (Sigma) and 0.05 mM 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (Sigma). Color was developed within 15-20 minutes and the reaction was stopped with addition of distilled water.

RESULTS

The open reading frame of *bipD* (933 bp) was amplified from genomic DNA of *B. pseudomallei* and sequenced (submitted to GenBank, accession no. EF120623). The nucleotide sequence of the PCR product was analyzed by BLAST and the Clustal X program showed 99% similarity to *B. pseudomallei* K96243. The amino acids of *B. pseudomallei* K96243 that differed from the deduced amino acids of this clinical strain were A34E, V292M and T303A (Fig 1). The deduced BipD protein (310 amino acid) is expected to have MW of 34 kDa. SDS-PAGE profile of the expression and purification of GST-BipD protein showed that it had an approximate molecular mass of~60 kDa consisting 34 kDa BipD and 26 kDa GST following cleavage with thrombin (Fig 2).

Immunoblot of crude GST-BipD showed a specific band when reacted with pooled absorbed melioidosis serum, whereas no band was detected when incubated with pooled absorbed normal serum or serum from patients with other bacterial infections (Fig 3A). As the crude fusion protein produced many

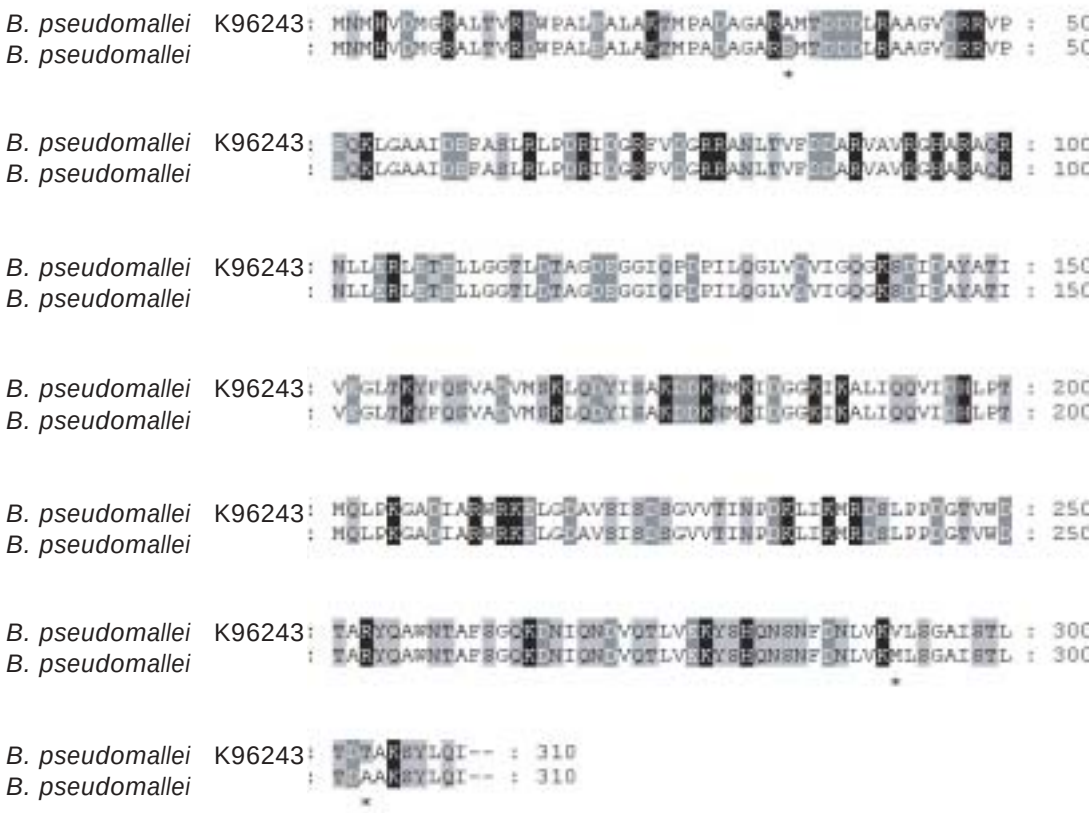

Fig 1–Alignment of amino acid sequence of BipD polypeptide of *B. pseudomallei* K96243 and *B. pseudomallei* experimental strain. The polypeptide has 310 amino acids. The asterix shows the deduced BipD amino acid sequence differences between *B. pseudomallei* K96243 and *B. pseudomallei* experimental strain. The black shade display basic amino acid group, and dark gray and light gray show acidic amino acid group and polar amino acid group, respectively. White shade is non-polar amino acid group.

non-specific bands with unabsorbed pooled melioidosis serum (Fig 3A, lane 1), therefore the fusion protein was purified using Glutathione Sepharose 4 Fast Flow beads. The intensities of non-spcific bands were reduced when purified BipD was reacted with pooled melioidosis serum (Fig 3B, lane 1) and an intense single band was seen with absorbed pooled melioidosis serum (Fig 3B, lane 4).

The purified GST-BipD protein was then employed on the membrane as antigen to detect antibodies in individual clinical serum samples, including 27 serum samples from patients infected by *B. pseudomallei* and 90 serum samples from non-melioidosis patients. Purified GST-BipD protein reacted with 21 of the 27 samples from patients previously diagnosed with melioidosis while 81 of the 90 serum samples from non-melioidosis patients gave no reaction, giving rise to sensitivity of 78 % and specificity of 90 % (Table 1). As these values are not high enough to satisfactorily identify melioidosis patients, GST was cleaved off and the experiments on clinical sera were repeated using purified BipD protein (0.04 µg). Sensitivity and specificity was 100% and 91.1%, respectively.

DISCUSSION

Sensitivity of 100% was obtained with

Table 1
Reactivity of GST-BipD protein and purified BipD protein to clinical serum by immunoblotting assay.

Test result	GST-BipD fusion protein		BipD protein	
	Melioidosis serum	Non-melioidosis serum	Melioidosis serum	Non-melioidosis serum
No of positive	21	9	27	8
No of negative	6	81	0	82
Total	27	90	27	90

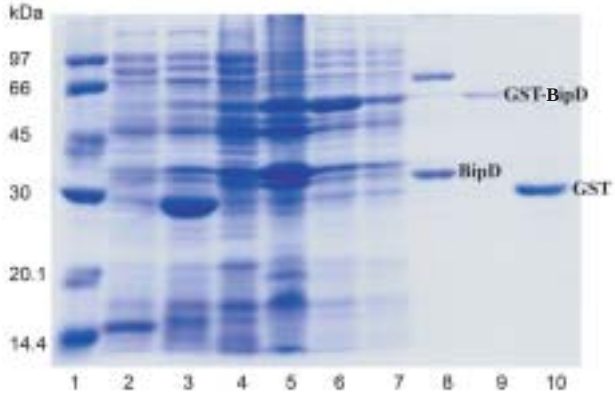

Fig 2–SDS-PAGE profile of GST-BipD recombinant plasmid pGEX 4T-1 containing fusion protein GST-BipD was expressed in *E. coli* BL21. Lane 1, protein molecular weight markers; lanes 2 and 3, protein from non-induced and induced *E. coli,* respectively; lane 4, non-induced transformed *E. coli*; lane 5, induced transformed *E. coli*; lane 6, crude GST-BipD protein preparation before being bound to Glutathione Sepharose 4 Fast Flow beads; lane 7, unbound proteins to beads, lane 8, fusion protein digested with thrombin; lanes 9 and 10, purified fusion protein and GST, respectively eluted from beads with 10 mM reduced glutathione.

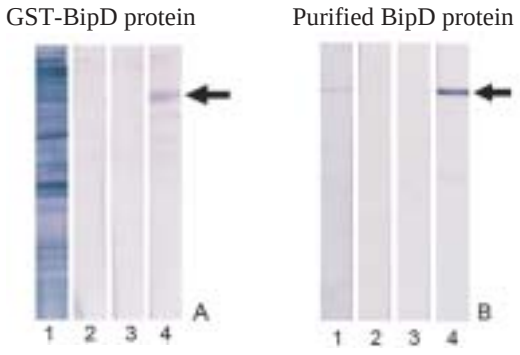

Fig 3–Immunoblotting of crude (A) and purified fusion protein (B). GST-BipD fusion protein and purified BipD protein were reacted with unabsorbed pooled melioidosis sera (lane 1A and 1B respectively), absorbed pooled normal sera (lane 2A and 2B), absorbed pooled sera from other bacterial infections (lane 3A and 3B) and absorbed pooled melioidosis sera (lane 4A, 4B). Arrow shows band specific to BipD protein.

BipD protein cleaved from GST fusion product in immunoblot detection of antibodies from clinical sera. The increase in sensitivity of the BipD after the GST was removed may be due to the presence of the GST that prevents binding of BipD to antibodies. However, the specificity of the BipD antigen with sera from non-melioidosis patients was not different from that of the fusion BipD protein. GST fusion BipD reacted with 9 other patients serum groups, namely, *Enterobacter aerogenes* bacteremic patients, 1 *Staphylococcus aureus* bacteremic patient, 1 *Streptococcus faecalis* bacteremic patient, 1 TB patient and 3 blood bank donors. However, using purified BipD protein, one of previously positive blood bank donor sample become negative. These false positive results of BipD immunoblotting may be caused by cross-reaction with antigenic BipD protein and

the high background of melioidosis infection. This high background of melioidosis antibodies in clinical sera need to be further evaluated if BipD protein is to be developed as a diagnostic test for melioidosis in endemic areas such as Thailand.

When we compared our results with the immunoglobulin M (IgM) and IgG rapid cassette test and a rapid immunochromogenic cassette test (ICT) (Wuthiekanum *et al*, 2004; Cheng *et al*, 2006), we have obtained a higher percent sensitivity and specificity. However, further evaluation of the sensitivity and specificity using IgM and IgG sera should be performed. In addition, it may also be possible to develop a monoclonal antibody against BipD antigen to detect BipD protein directly from clinical specimens. This approach may allow early detection of melioidosis and eliminate the problem of high background antibodies of melioidosis in endemic area.

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