EXPRESSION AND PURIFICATION OF 30 KILODALTON PROTEIN ANTIGEN OF ARA$^-$ *BURKHOLDERIA PSEUDOMALLEI*

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Abstract. The 30 kDa protein of *B. pseudomallei* is found in virulent Ara$^-$ but not avirulent Ara$^+$ strain. The gene was cloned in *Escherichia coli* JM105 employing pInIII-C2 vector. The open reading frame was 870 nucleotides with a guanine plus cytosine content of 69.9%. Arginine was the most abundant amino acid in the protein, having a PI of 12.65. Nucleotide sequence of the gene was 96% identical to *B. pseudomallei* 1710b chromosome II sequence CP000125.1, encoding an oxidoreductase of the short chain dehydrogenase/reductase family. The 30 kDa antigen was expressed as a maltose-fusion protein with a yield of 5.25 mg/l of bacterial culture.

INTRODUCTION

Melioidosis is an infectious disease caused by *Burkholderia pseudomallei*. It is endemic in Southeast Asia and northern Australia, with report of annual incidence of 4.4 cases per 100,000 in Ubon Ratchathani Province in Northeast Thailand (Suputtamongkol *et al*, 1994). Sporadic cases of melioidosis have also been reported in areas outside Asia (Dance, 1991, 2000). When infection occurs, a wide variety of outcome can be found, ranging from asymptomatic state with positive specific antibodies (Howe *et al*, 1971) to a variety of clinical manifestations, including pneumonia, genitourinary infection, abscess, bacteremia, septic shock and death (Cheng and Currie, 2005). At present, there is no vaccine available for the prevention of this disease (Sprague, 2004; Cheng and Currie, 2005).

Absolute diagnosis of melioidosis is the isolation of *B. pseudomallei* from body fluid of the patients although many rapid diagnostic tests, both antigen or antibody detection based, and molecular diagnosis have been reported (Ashdown *et al*, 1989; Kunakorn *et al*, 1990; Petkanjanapong *et al*, 1992; Anuntagool *et al*, 1993, 2000; Dharakul *et al*, 1997; Pongsunk *et al*, 1999; Cheng and Currie, 2005), only a few have been extensively tested in the field.

We have produced monoclonal antibody (MAb) specific to a 30 kDa protein antigen of *B. pseudomallei* for the detection of the organisms in clinical specimen (Pongsunk *et al*, 1999; Ekpo *et al*, 2007). This MAb reacted with only Ara$^-$ but not Ara$^+$ strain of *B. pseudomallei*. Sensitivity of a latex agglutination test using this monoclonal antibody is 100% with specificity greater than 85% (Pongsunk *et al*, 1999; Ekpo *et al*,

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2007). This MAb could neutralize cytotoxic activity of crude culture filtrate of *B. pseudomallei*. Thus, this 30 kDa protein of *B. pseudomallei* appears to be a promising candidate protein for use in the development of diagnosis kit of melioidosis, in study of pathogenesis or even possibly in vaccine development.

In this study, we report the synthesis and characterization of this 30 kDa antigen protein of Ara$^-$ *Burkholderia pseudomallei*.

MATERIALS AND METHODS

Bacterial strains

Clinical *B. pseudomallei* isolates (Ara$^-$; L-arabinose non-assimilation) were obtained from patients admitted to Khon Kaen Regional Hospital, Khon Kaen Province, and Sappasitprasong Hospital, Ubon Ratchathani Province, in the northeastern part of Thailand. Nine Ara$^+$ (L-arabinose assimilation) soil isolates and 10 Ara$^-$ soil isolates were kindly provided by Prof Dr Stitaya Sirisinha, Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand. Four strains of *B. mallei* (ATCC 2344, ATCC 10399, ATCC 15310, and NCTC 120) were provided by Dr Takayuki Ezaki, Department of Microbiology, Gifu Medical School, Gifu, Japan. Other bacteria used in this study were isolated from clinical specimens obtained from patients admitted to the two above mentioned hospitals and from Siriraj Hospital, Mahidol University, Bangkok, Thailand. *Escherichia coli* JM 105 and JM 107 (kindly provided by Dr T Ezaki) used as the host strains for transformation were cultured in Luria Bertain (LB) medium (Difco, USA).

Monoclonal antibody (MAb) and latex agglutination assay

MAb (IgMκ) against the 30 kDa protein of crude culture filtrate (CCF) and outer membrane protein (OMP) antigens of *B. pseudomallei* was produced as previously described (Pongsunk *et al*, 1999). MAb-sensitized latex particles were prepared and latex agglutination with bacterial colonies was performed as previously described (Pongsunk *et al*, 1999). In brief, a small portion of a colony suspended in normal saline solution was placed on glass slide and mixed with the latex reagent. Agglutination was detected visually after rotation for at least 2 minutes.

DNA preparation, library construction and expression screening of *B. pseudomallei* library

Genomic DNA of *B. pseudomallei* was prepared by using guanidine thiocyanate method (Maniatis *et al*, 1982). DNA was partially digested with *Sau*3Al and fragments ranging from 1.5 to 6.0 kb were size-fractionated by electrophoresis in 0.7% agarose. DNA was purified using an Elutip-d column (Schleicher and Schuell, German) and inserted into *Bam*HI site of pIn-IIICI, pIn-IIIC2 and pIn-IIIC3 plasmid vectors (Inouye, 1983). Recombinant plasmids were transfected into competent *E. coli* JM 107 and screened with the MAb as previously described (Sukosol *et al*, 1993).

DNA sequencing

DNA insert in pIn-IIIC was subcloned into pUC18 and pUC19 for nucleotide sequencing. Both strands were sequenced using dideoxy chain termination method of Sanger *et al* (1977) employing a modified T7 DNA polymerase ("Sequenase", US Biochemicals, USA). Sequences were analyzed with BLAST and ENTREZ network programs (GenBank, National Center for Biotechnology Information). The nucleotide sequence obtained was deposited in GenBank database, accession no. AF 139591.

Construction of mal E gene fusion

B. pseudomallei gene encoding the 30 kDa protein antigen was amplified by PCR

using primers based on the nucleotide sequence. Primers included *Bam*HI and *Xba*I at the 5' end and 3' end respectively. DNA template was prepared by mixing a loop of bacterial cells grown on LB agar plate with 100 µl of distilled water and heating for 5 minutes in boiling water. The 100 µl PCR reaction mixture consisted of 5 µl DNA template, 150 mM of each primer, 200 µM nucleotide mixture (Ultrapure deoxynucleoside triphosphate set, Pharmacia Biotechnology LKB, USA), 10 µl of 10X *Taq* polymerase buffer (containing 500 mM KCl, 100 mM Tris- HCl pH 8.3, 15 mM $MgCl_2$ and 0.01% gelatin), and 5 units of *Taq* polymerase (Perkin-Elmer, USA). PCR amplification was carried out using a Perkin-Elmer Cetus DNA Cycler 480 (Perkin-Elmer). After an initial denaturation step of 2 minutes at 95ºC, the amplification was performed for 35 cycles, each consisting of 95ºC for 1 minute, 68ºC for 1 minute and 72ºC for 3 minutes, and then 10 minutes at 72ºC for the final extension step.

DNA fragment of the PCR was isolated from 1.0% agarose gel using Sephaglas (Pharmacia, USA) and digested with *Bam*HI and *Xba*I. Digested DNA was then ligated to the *Bam*HI - *Xba*I site of plasmid pMAL-c2 (New England Biolabs, USA) overnight at 16ºC. Recombinant plasmid was transfected into competent *E. coil* JM 105 and screened with the MAb (Sukosol *et al*, 1993).

Expression and purification of the fusion protein

An overnight culture of transfected *E. coli* containing the recombinant plasmid was inoculated into 2xYT medium containing 2 mM glucose, 250 µg/ml ampicilin and 100 µg/ml streptomycin. The bacterial culture was allowed to grow at 30ºC with good aeration until A_{600} reach 0.5. An aliquot of 1 ml was centrifuged for 2 minutes at 3,000*g* (Tomy Seiko, Tokyo, Japan). The supernatant was discarded and the cells were resuspended in 50 µl of SDS-PAGE sample buffer. This sample was used as uninduced cells. Isopropylthiogalactoside (IPTG) (Sigma, USA) was added to the remaining culture to give a final concentration of 1 mM. After further incubation at 30ºC for 3 hours, an aliquot of 0.5 ml was centrifuged for 2 minutes. The pellet was resuspended in 100 µl of SDS-PAGE sample buffer. This sample was used as induced cells.

The remaining culture was centrifuged for 10 minutes at 4,000*g* (Tomy Seiko, Tokyo, Japan). The pellet was resuspended in 10 ml of column buffer (20 mM Tris HCl, 200 mM NaCl, 1 mM EDTA), frozen at -20ºC overnight, then thawed in cold water, sonicated in short pulses of 15 seconds, and centrifuged at 9,000*g* for 20 minutes at 4ºC. The supernatant, named the crude extract, was saved on ice for further purification. The pellet was resuspended in column buffer. It was named insoluble matter and kept at -20ºC for further analysis.

The affinity column for the fusion protein purification was prepared by pouring amylase resin (New England Biolabs) into a 50 ml syringe plugged with silanized glass wool. After the column was washed with 8 column volumes of column buffer, crude extract was loaded onto the column at a flow rate of 1 ml/minute. The column was washed with 10-12 column volumes of column buffer. The fusion protein was then eluted with column buffer containing 10 mM maltose and 3 ml fractions were collected. Fusion protein was detected by measuring UV absorbance at 280 nm. Protein-containing fractions were pooled and concentrated by ultrafiltration using a membrane with MW cut-off of 10,000 (Sartorius AG, Goettingen, Germany). Protein concentration was determined by using a commercial protein assay kit (BioRad Laboratories, Richmond, CA).

SDS-PAGE

The protein profiles of bacterial antigens were examined by SDS-PAGE in a minigel apparatus (Bio-Rad Laboratories) (Pongsunk *et al*, 1999). A 3.5% stacking and a 12% separating acrylamide gel were used. A sample was solubilized under denaturing condition by using sample buffer containing 0.0625 M Tris-HCl pH 6.8, 1% SDS, 10% glycerol, 5% 2-mercaptoethanol and bromphenol blue, and heated in a boiling water bath for 5 minutes before being loaded onto the gel. The electrophoresis was performed at a constant current of 170 mA, until the bromphenol blue reached the bottom edge of the gel. After that, the separated polypeptide bands were visualized by staining with Coomassie blue R-250.

RESULTS

Determination of presence of 30 kDa protein in Ara$^+$ and Ara$^-$ *B. pseudomallei*

To determine the cell-associated form of 30 kDa protein in *B. pseudomallei* strains, both Ara$^+$ and Ara$^-$ strains, and in other bacteria, reversed passive agglutination, using latex particles coated with the MAb specific to the 30 kDa protein, was performed with bacterial colonies. All Ara$^-$ *B. pseudomallei* strains gave strong positive agglutination, whereas no reaction was seen with the 10 Ara$^+$ *B. pseudomallei* strains (Table 1). Moreover, no cross-reactivity with other gram-negative bacteria was observed, except in the case of the closely related species, *B. mallei*. However, with gram-positive bacteria, weakly

Table 1
Reversed passive agglutination of Ara$^-$, Ara$^+$ *B. pseudomallei* and other bacterial colonies using latex reagent coated with MAb specific to the 30 kDa protein of *B. pseudomallei*.

Bacteria	No. positive reaction/no. tested
B. pseudomallei (Ara$^-$ isolated from clinical specimens)	100/100
B. pseudomallei (Ara$^-$ isolated from soil)	9/9
B. pseudomallei (Ara$^+$ isolated from soil)	0/10
Acinetobacter spp	0/15
Aeromonas spp	0/5
B. cepacia	0/2
B. mallei	4/4
Escherichia coli	0/15
Klebsiella spp	0/13
Proteus spp	0/10
Providencia spp	0/1
Pseudomonas spp	0/16
Salmonella spp	0/16
Vibrio spp	0/2
Xanthomonas spp	0/6
Bacillus cereus	1/1[a]
Staphylococcus spp	0/5
Streptococcus spp	4/14[a]

[a]Weakly positive reaction

positive reaction was observed with one isolate of *B. cereus* and a number of *S. pyogenes* isolates.

Cloning and DNA sequencing of 30 kDa protein gene of *B. pseudomallei*

Approximately $3x10^4$ clones containing *B. pseudomallei* genomic DNA were plated and protein expression was analyzed using the MAb. A higher number of positive clones were found in transformants containing recombinant pInIII-C2 than in the other two vectors. A representative positive clone was characterized by DNA sequencing. The open reading frame (ORF) contained 870 nucleotides with a *Sal*I restriction site at nucleotide position 104. The guanine (G) plus cytosine (C) content of the gene was 69.9%. Arginine was the most abundant amino acid in the protein (60 Arg residues among 98 amino acids) giving rise to PI of 12.65. Comparison of the amino acid and nucleotide sequences with sequences in GenBank, National Center for Biotechnology Information, revealed nucleotide sequence identity of 96% with *B. pseudomallei* 1710b chromosome II ORF CP000125.1, encoding oxidoreductase of the short chain dehydrogenase/reductase family.

Expression and purification of fusion protein

In order to express the 30 kDa protein in the pMAL-C2 vector, PCR was used to generate the nucleotide sequence. Fragment of the expected size was cloned into the pMAL-C2 expression vector. Transformed *E. coli* expressing *B. pseudomallei* 30 kDa protein was identified by colony staining with MAb. The fusion protein was expressed and purified using an affinity column. Fig 1 shows SDS-PAGE profiles of the fusion protein, MW of 73 kDa. The maltose binding protein has a MW of 42.7 kDa. After purification, the fusion protein was seen as a single band in the SDS-PAGE stained with Coomassie brilliant blue. The concentration of the fusion protein determined by using a commercial protein assay kit (BioRad Laboratories, Richmond, CA) was 5.25 mg/l of bacterial culture.

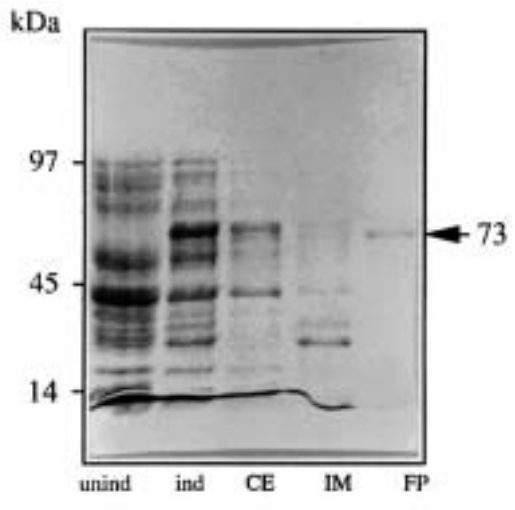

Fig 1–SDS-polyacrylamide gel electrophoresis of proteins from transfected *E. coli*. Ten microliters of each bacterial sample was loaded onto 12% acrylamide gel. The electrophoresis was performed at a constant current of 170 mA, and after that, the gel was stained with Coomassie blue R-250. Lane 1, uninduced cells (unind); lane 2, induced cells (ind); lane 3, crude extract (CE); lane 4, insoluble matter (IM); lane 5, affinity purified fusion protein (FP) eluted from amylose column.

DISCUSSION

B. pseudomallei is saprophytic organism that can survive in a variety of hostile environmental conditions (Cheng and Currie, 2005). Various components of the bacteria have been reported to be virulence factors, including glycocalyx polysaccharide capsule (Steinmetz *et al*, 1999) and type III secretion systems (TTSS) (Rainbow *et al*, 2002). The

loss of the ability to assimilate L-arabinose (Ara^-) is linked to increased virulence of *B. pseudomallei* in humans and animal (Powell *et al*, 2003).

We have produced MAb specific to 30 kDa protein that has been found to react only with Ara^- *B. pseudomallei* (Pongsunk *et al*, 1999; Ekpo *et al*, 2007). We have cloned and expressed in *E. coli* the *B. pseudomallei* 30 kDa protein using pMAL-C2 expression vector. The G+C content of the gene is high (69.9%), characteristic of *B. pseudomallei* genome.

This 30 kDa protein, the major protein present in the bacterial culture supernatant and on the surface of the virulent Ara^- *B. pseudomallei* (Kunakorn *et al*, 1990) will be valuable for further studies on diagnosis and pathogenesis of melioidosis. Based on sequence identity, 30 kDa protein has significant similarity to oxidoreductase of short chain dehydrogenase/reductase (SDR) family. The oxidoreductase is a group of enzyme that utilize NADP or NAD to catalyse an oxidation-reduction reaction. There are over 3,000 members in the SDR family, extending from bacteria and archaea to human (Keller *et al*, 2006). The enzymes have a wide range of substrate spectrum, ranging from steroids, alcohols, sugars, and aromatic compounds to xenobiotics (Kallberg *et al*, 2002). The characteristics of *B. pseudomallei* 30 kDa protein should be further studied to obtain more understanding the role of this enzyme in the pathogenic processes of Ara^- *B. pseudomallei*.

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