SEROLOGICAL DIAGNOSIS

Cloning and characterisation of *malE* in *Burkholderia pseudomallei*

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No recombinant protein is available for serodiagnosis or skin test in the diagnosis of melioidosis. This report describes the cloning of the *malE* gene, which encodes an immunogenic protein of *Burkholderia pseudomallei*. Bi-directional DNA sequencing of *malE* revealed that the gene contained a single open reading frame encoding 416 amino acid residues with a predicted molecular mass of 44.4 kDa. BLAST analysis showed that the putative protein encoded by *malE* is homologous to the maltose-binding protein (MBP) of other bacteria. It has 48% and 63% amino acid identity and similarity with the MBP of *Brucella abortus*, and *malE* complementation assay showed that it partially complemented the function of the MBP of *Escherichia coli*. Several highly conserved regions among the MBP of *B. pseudomallei*, *B. abortus*, *Salmonella enterica* serotype Typhimurium, *E. coli* and *Enterobacter aerogenes* were observed. These regions represent signatures A, B, C, D and F identified in the MBP of *E. coli*. Further sequence analysis revealed that the first 24 amino acid residues of the MBP of *B. pseudomallei* probably represent the N-terminal signal peptide of the protein. Similar to the signal peptide of the MBP of *E. coli*, *Ent. aerogenes* and *S. Typhimurium*, the MBP of *B. pseudomallei* contains two basic residues in the first eight amino acids, followed by a hydrophobic core, with the last three amino acids in the signal peptide being Ala-Gln-Ala, conforming to the consensus sequence Ala-X-Ala at positions −3 to −1 relative to the site of proteolytic cleavage for recognition by signal peptidase I. Further studies on serodiagnosis of melioidosis with recombinant MBP should be performed.

Introduction

Melioidosis is a serious human disease, endemic mainly in south-east Asia, caused by *Burkholderia pseudomallei*. Human cases have occurred throughout the world between latitudes 20° north and south [1]. *B. pseudomallei* is a natural saprophyte that can be isolated from soil, stagnant streams, rice paddies and ponds, which are the major natural reservoirs of the bacteria [2]. Illness can be manifested as an acute, subacute, or chronic process. Moreover, the incubation period of melioidosis can vary from 2 days to 28 years [3].

Definitive diagnosis of melioidosis depends on the isolation and identification of *B. pseudomallei* from blood, sputum, pus, swabs and other clinical specimens. Despite this, the number of *B. pseudomallei* in clinical specimens collected from non-bacteremic patients is often low. Furthermore, laboratory identification is often delayed because laboratory personnel are unfamiliar with the bacteria and *B. pseudomallei* may be overlooked as ‘*Pseudomonas* species’ [4]. Therefore, serological tests are needed to detect the antibody response of patients with melioidosis. An indirect haemagglutination assay (IHA), complement fixation assay, indirect immunofluorescent assay and enzyme-linked immunosorbent assay (ELISA) have been described, and IHA and ELISA are the most commonly used methods [5–8]. IHA is observer-biased, and a 16-fold variation in serological titre is not uncommon due to the variability of lipopolysaccharide antigen coating on red cells. Recently, it has been shown that ELISA with lipopolysaccharide is superior to IHA in terms of both sensitivity and specificity for the diagnosis of melioidosis [6]. However, the test is not specific in the discrimination between past and active infections. Therefore, improved antibody detection might be
achieved if a recombinant antigenic protein was available. This study reports the cloning of the mae gene in *B. pseudomallei*, which encoded a protein immunogenic in guinea-pigs and potentially suitable as a diagnostic antigen.

**Materials and methods**

**Bacterial strain and growth conditions**

The *B. pseudomallei* strain used was isolated from a blood culture from a patient suffering from melioidosis. The bacteria were grown on blood agar plates at 37°C to obtain single bacterial colonies, which were then cultured in trypticase soy broth at 37°C for 24 h.

**Generation of antibodies**

To produce a polyclonal guinea-pig antibody, *B. pseudomallei* suspended in phosphate-buffered saline (PBS) with phenol 0.05% at a turbidity of McFarland standard 3 and mixed with an equal volume of complete Freund’s adjuvant was injected intramuscularly into the thigh of a guinea-pig. Incomplete Freund’s adjuvant was used in subsequent immunizations, and a total of three inoculations were completed in 2 weeks. Blood was collected from the guinea-pig 1 week after the last immunisation dose. The serum obtained was used for screening the expression library.

**Cloning of the mae gene**

Total genomic DNA was obtained from 100 ml of a *B. pseudomallei* culture according to a standard protocol [9]. The genomic DNA was partially digested with *SalI* (Boehringer Mannheim, Germany). Fragments of sizes 1.5–6 kb in the partial digest were then ligated to the *BamHI* site of the vector provided by the ZAP Express Vector Kit, and a phage expression library was constructed according to the manufacturer’s instructions (Strategene, CA, USA). The library had at least one million independent phage plaques, with >95% containing inserts of an average size of 2.3 kb, as checked by PCR amplification of 100 clones with T3 and T7 primers.

Approximately 50,000 plaques of this library were screened with guinea-pig anti-*B. pseudomallei* antiserum at a 1 in 1000 dilution according to the manufacturer’s instructions. Briefly, the library was plated on NZY – NaCl 0.5% w/v, MgSO4·7H2O 2% w/v, yeast extract 5% w/v, NZ amine (casein hydrolysate) 10% w/v, pH 7.5 – plates at 5000 plaques/plate with 600 µl of XL1-Blue cells at an OD600 of 0.5 and 6.5 ml of NZY top agar. The plates were incubated at 37°C for 8 h and the proteins were transferred to nitrocellulose membranes. After blocking with bovine serum albumin (BSA) 3% and skimmed milk 7% in PBS, the membranes were incubated with guinea-pig anti-*B. pseudomallei* antiserum at a dilution of 1 in 1000 at 25°C for 1 h. After washing three times with BSA 3% in PBS, the membranes were incubated with rabbit anti-guinea-pig antibody conjugated with horseradish peroxidase (Zymed Laboratories, South San Francisco, CA, USA) at a dilution of 1 in 5000 for 1 h. After washing three times with BSA 3% in PBS, antigen–antibody interaction was detected with the ECL fluorescence kit (Amersham Life Science, Bucks). Ten positive phage clones were isolated, and their DNA inserts were excised with ExAssist helper phage in SOLR™ cells – el4 (McrA+) Δ(incCB-ksdSMR-mrr) 171 sbcC recB recC wrvC umuC. Tn5 (Kan‘) lac glyrA96 relA thi-1 endA1 A8 [F’ proA8 lacZAM15]

Western blot analysis

Overnight cultures of SOLR cells with pBK-CMV and SOLR cells with pBK-CMV-mae were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 4 h. The cells were centrifuged at 13,000 rpm for 5 min and resuspended in PBS with Tween 20 1% v/v and 0.5 mM phenylmethylsulphonyl fluoride. Then, 25 µl of the cell extracts obtained were electrophoresed on an SDS-polyacrylamide 10% gel and electrophotobloted on to a nitrocellulose membrane (BioRad, Hercules, CA, USA). The blot was incubated with a 1 in 1000 dilution of pre-immune guinea-pig serum and serum of a guinea-pig immunised with *B. pseudomallei*, and antigen–antibody interaction was detected with the ECL fluorescence kit (Amersham Life Science).

**DNA sequencing**

DNA sequencing was performed with primer sets of pBK-CMV (T3 and T7) and synthetic primers. Bi-directional DNA sequencing was performed with an ABI automatic sequencer according to the manufacturer’s instructions (Perkin-Elmer, USA). The DNA sequence was analysed by BLAST Search with the National Center for Biotechnology Information server at the National Library of Medicine (Bethesda, MD, USA). The searches were performed at both the protein and DNA levels. Phylogenetic trees were constructed by the Clustal method with MegAlign™ 4.00 (DNA star, WI, USA).

**PCR amplification of mae from 12 other strains of B. pseudomallei and other gram-negative bacteria**

All bacterial strains were isolated from hospitalised patients. Bacterial DNA extraction was modified from a published protocol [10]. 80 µl of 0.05 M NaOH was added to 20 µl of bacterial cells suspended in distilled water and the mixture was incubated at 60°C for 45 min followed by addition of 6 µl of Tris-HCl (pH 7.0), achieving a final pH of 8.0. The resultant mixture was diluted 100-fold and 5 µl of the diluted extract was
used for PCR. Bacterial DNA extracts from 12 other strains of *B. pseudomallei*, *Pseudomonas aeruginosa* (two strains), *Escherichia coli* (one), *B. cereus* (one), *B. picketti* (one) and control were amplified with 0.5 µM primers (LPW77 5’-ATGAAAATTCGGCC
GATC3’ and LPW78 5’-CGTTGCGTTACTTCCACCTT-
3’) (Gibco-BRL, USA). The PCR mixture (50 µl) contained bacterial DNA, PCR buffer (10 mM Tris-
HCl, pH 8.3, 50 mM KCl, 3 mM MgCl₂ and gelatin
0.01%), 200 µM of each dNTP and Taq polymerase
(Boehringer Mannheim) 1.0 U. The mixtures were amplified in 40 cycles of 94°C for 1 min, 55°C for
1 min and 72°C for 2 min, and a final extension of
72°C for 10 min in an automated thermal cycler
(Perkin-Elmer Cetus, Gouda, The Netherlands). Dis-
tilled water was used as the negative control. A 10-µl
volume of each amplified product was electrophoresed
in agarose 1.0% w/v gel, with a molecular size marker
(ΦX-174 DNA HaeIII digest, Boehringer Mannheim)
in parallel. Electrophoresis in Tris-borate-EDTA buffer
was performed at 100 V for 1.5 h. The gel was stained
with ethidium bromide (0.5 µg/ml) for 15 min, rinsed
and photographed under UV light.

**MalE complementation assay**

*E. coli* LH1245 (MC4100 malE::Tn5 malTp1p7) was a
gift from Dr Howard Shuman [11]. Samples
(7 x 10⁸ cfu) of wild-type *E. coli* ATCC 25922, *E.
coli* LH1245 transformed with pBK-CMV and *E. coli*
LH1245 transformed with pBK-CMV-malE from over-
night culture broth were each inoculated into 200 µl
of broth medium (containing maltose 1% w/v, peptone
1% w/v, NaCl 0.5% w/v and Andrade’s indicator; five
wells each) in a microtiter plate. After incubation
for 24 h, the absorbance of each well was measured at
547 nm, with uninoculated broth as a blank. The mean
absorbance of the wells containing wild-type *E. coli*, *E.
coli* LH1245 transformed with pBK-CMV and *E. coli*
LH1245 transformed with pBK-CMV-malE were com-
pared by one-way ANOVA; p <0.05 was regarded as
statistically significant.

**Nucleotide sequence accession number**
The nucleotide sequence of the *malE* gene of *B.
pseudomallei* has been deposited with GenBank under
accession no. AF274304.

**Results**

**Cloning of malE**

An animal hyperimmune serum was generated by
immunising a guinea-pig with killed *B. pseudomallei*
cells. About 50 000 independent phage plaques were
screened with this guinea-pig hyperimmune serum. Ten
positive plaques were selected, purified and converted
into plasmids. When induced with isopropyl-β-D-
thiogalactopyranoside, two of the 10 isolates produced
protein bands of 44.4 kDa that were recognised by the
guinea-pig hyperimmune serum on a Western blot (Fig.
1). PCR and sequence analysis of the two clones revealed
a single open reading frame of c. 12.5 kb.

**Sequence analysis of malE**

Bi-directional DNA sequencing of the insert revealed
that the DNA contained a single open reading frame
encoding 416 amino acid residues with a predicted

![Fig. 1. Western blot analysis of MBP of *B. pseudomallei*. Cell extracts of overnight cultures of SOLR cells with pBK-
CMV (lane 1, uninduced (U)); 2, induced (I) and SOLR cells with pBK-CMV-malE (3, uninduced (U); 4, induced (I))
electrophoresed on an SDS-polyacrylamide 10% gel. A band of c. 44.4 kDa can be detected (lanes 3 and 4). Antigen–
antibody interaction was detected with serum from a guinea-pig immunised with *B. pseudomallei* (lanes 11 and 12),
but not with pre-immune serum (5–8).](image-url)
Fig. 2. DNA and amino acid sequences of MBP of *Pseudomonas*; *mae* DNA contains a single open reading frame encoding 416 amino acid residues with predicted molecular mass of 44.4 kDa. The N-terminal cleavable signal peptide of 24 amino acids is underlined. The Shine-Dalgarno (S.D.) sequence is shaded in grey.
molecular mass of 44.4 kDa. The DNA and predicted protein sequences are shown in Fig. 2.

BLAST analysis to search for homologues that might suggest the potential biological functions revealed that the putative protein encoded by the gene is homologous to the maltose-binding protein (MBP) of other bacteria (Fig. 3a). It has 48% and 63% amino acid identity and similarity with the MBP of Brucella abortus (GenBank accession no. O06875), 21% and 36% amino acid identity and similarity with the MBP of Salmonella enterica serotype Typhimurium (GenBank accession no. P19576), 22% and 36% amino acid identity and similarity with the MBP of E. coli (GenBank accession no. P02928), and 21% and 35% amino acid identity and similarity with the MBP of Enterobacter aerogenes (GenBank accession no. P18815). The gene was named malE.

Several highly conserved regions among the MBP of B. pseudomallei, Br abortus, S. typhimurium, E. coli and Ent. aerogenes were observed (Fig. 4). These regions represented signatures A, B, C, D and F identified in the MBP of E. coli [12].

PCR amplification of malE from 12 other strains of B. pseudomallei and other gram-negative bacteria

PCR amplification of the malE gene showed a band of 1275 bp in all 12 other strains of B. pseudomallei, but not in the two strains of P aeruginosa, one strain of E. coli, one strain of B. cepacia and one strain of B. picketti (Fig. 5).

MalE complementation assay

The mean absorbance of the wells inoculated with wild-type E. coli (1.22) was significantly higher than those inoculated with E. coli LH1245 transformed with pBK-CMV (9.17 × 10⁻²) and E. coli LH1245 transformed with pBK-CMV-malE (0.43). The mean absorbance of the wells inoculated with E. coli LH1245 transformed with pBK-CMV-malE was significantly higher than those inoculated with E. coli LH1245 transformed with pBK-CMV, showing that the MBP of B. pseudomallei can partially complement the function of the MBP of E. coli.

Discussion

The only MBP with confirmed sequence and function were cloned from E. coli, S. Typhimurium and Ent. aerogenes in 1984, 1989 and 1989, respectively [13, 14]. In 1997, Denoel et al. discovered a T-cell dominant antigen of 39 kDa (P39) from Br abortus [15]. However, its function remained unknown until 1999, when de Fays et al. used a combination of sequence alignments, consensus of secondary structure predictions, and fold recognition tools to improve the modelling system of P39, thereby suggesting that P39 was indeed the MBP of Br. abortus [12]. Furthermore, analysis of the sequences downstream to P39 in Br. abortus revealed the presence of two open reading frames that are highly homologous to malE and malG of E. coli, which encode the integral inner-membrane components of binding protein-dependent transporters with the highly conserved EAA loop, and these two proteins are also located downstream to the malE gene.

![](Phylogenetic_tree_B. pseudomallei.png)

**Fig. 3.** Phylogenetic tree, based on known bacterial MBP amino acid sequences (**a**) and their corresponding 16S ribosomal RNA gene sequences (**b**), illustrating the position of *B. pseudomallei*. This figure provides an overview of the evolutionary relationships among these bacteria, highlighting B. pseudomallei's close relatives and its potential role in the malE gene family. (334 P.C.Y. Woo et al.)
Fig. 4. Amino acid sequence alignment of the MBP of B. pseudomallei (BP), Br. abortus (BA), S. Typhimurium (ST), E. coli (EC) and Ent. aerogenes (EA). Signatures A, B, C, D and F and the signal peptides are shaded in grey, with the amino acid residues that are identical between the MBP of B. pseudomallei and those of any of the other four bacteria in these regions in bold type.
in *E. coli*. The present study cloned and sequenced a 44.4-kDa immunogenic protein of *B. pseudomallei* identified with guinea-pig hyperimmune serum. Sequence analysis showed that it has 48% and 63% amino acid identity and similarity with P39 of *Br. abortus*. Furthermore, functional analysis by *malE* complementation assay showed that this protein partially complemented the function of MBP in *malE*-deficient *E. coli*. We therefore concluded that the 44.4-kDa protein is the MBP of *B. pseudomallei*.

The phylogenetic tree constructed by using the amino acid sequences of the MBP of *B. pseudomallei*, *Br. abortus*, *S. Typhimurium*, *E. coli* and *Ent. aerogenes* is similar to the one constructed using the 16S ribosomal RNA gene sequences of the corresponding bacteria (Fig. 3a and b), except that the relationship between the MBP of *B. pseudomallei* and that of *Br. abortus* is closer than the relationship between the 16S ribosomal RNA sequence of *B. pseudomallei* and that of *Br. abortus*, suggesting that the MBP of the two bacteria probably evolved from the same ancestor. When the sequences of the MBP of the five bacteria were aligned, it showed that there were five conserved regions. These five regions corresponded to signatures A, B, C, D, and F of the MBP of *E. coli* [12].

The first 24 amino acid residues of the MBP of *B. pseudomallei* probably represent the N-terminal signal peptide of the protein. Proteins destined for export across membranes are synthesised as precursors with N-terminal signal peptides. After translocation across the membrane, the signal peptide would be cleaved, releasing the mature protein. It has been shown that the signal peptide of the periplasmic MBP of *E. coli* demonstrated characteristics common to other signal peptides [16,17], which include three characteristic regions: the N-terminal eight amino acid residues, of which three are basic; this is followed by a hydrophobic core with a stretch of hydrophobic and neutral residues; and the sequence Ala-X-Ala, at positions –3 to –1 relative to the site of proteolytic cleavage for recognition by signal peptidase 1 [18,19]. Similar to the MBP of *E. coli*, the MBP of *Ent. aerogenes* and *S. Typhimurium* also contain the three characteristic regions, although in the signal peptide of *S. Typhimurium* only two of the first eight amino acid residues are basic. For the MBP of *B. pseudomallei*, there are also two basic residues in the first eight amino acids, followed by a hydrophobic core which is two amino acid residues shorter than those in *E. coli*, *Ent. aerogenes* and *S. Typhimurium* (Fig. 4). The last three amino acid residues in the signal peptide are Ala-Gln-Ala, instead of Ala-Leu-Ala in *E. coli*, *Ent. aerogenes* and *S. Typhimurium*.

We speculate that there is a guanosine missing from the *malE* gene sequence of *Br. abortus* [15]. In the MBP of *Br. abortus* (Fig. 4), no basic residues can be found in the first eight amino acids, and there are basic (lysine, arginine) and acidic (glutamic acid) amino acid residues following it, and there is no Ala-X-Ala sequence, irrespective of whether the ATG (as described in GenBank) or GTG (as described by Denuel et al [15]) is used as the putative start codon. Moreover, the amino acid sequence upstream to signature A in *Br. abortus* is 15–19 amino acid residues shorter than those of *B. pseudomallei*, *E. coli*, *Ent. aerogenes* and *S. Typhimurium*. Furthermore, during cloning of the gene, attempts to obtain the N-
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However, if a guanosine is added to the nucleotide sequence (Fig. 6, BA (New)), a frameshift will result in the first 25 amino acids of the MBP sequence having 50% amino acid identity with the corresponding region in B. pseudomallei (Fig. 6, BP), which is similar to the 48% amino acid identity between the two proteins in the rest of the sequence, in contrast to only 21% amino acid identity in this region between the two proteins if the original nucleotide sequence (Fig. 6, BA (GenBank)) is used (p < 0.05 by \( \chi^2 \) test). Furthermore, a stretch of hydrophobic and neutral amino acids that end in Ala-Asn-Ala, similar to the consensus sequence of the latter half of the signal peptide in the other four bacteria, would be present (Fig. 6, BA (New)).

In conclusion, the male gene which encoded the MBP in B. pseudomallei that is immunogenic in guinea-pigs was cloned, sequenced and characterised. Further experiments on its immunogenicity in man and studies on the serodiagnosis of melioidosis with MBP should be performed.

This work was partly supported by the Committee of Research and Conference Grants, University of Hong Kong, Hong Kong. We thank Dr Howard Shuman for providing E. coli LH1245.

References


