Sequencing and characterization of a novel serine metalloprotease from *Burkholderia pseudomallei*

May-Ann Lee *, Yichun Liu

*Defence Medical Research Institute, Clinical Research Centre, NUS, 10 Medical Drive #02-04, Singapore 117597, Singapore*

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Abstract

*Burkholderia pseudomallei*, a Gram-negative bacterium is found in the soil and water, mainly in Southeast Asia and Northern Australia. It is responsible for melioidosis in human and animals. The bacteria produce several potential virulent factors such as extracellular protease, hemolysin, lipase and lecithinase. The isolation of virulence genes and the study of their functions will contribute to our understanding of bacterial pathogenesis. Previous studies have implicated protease as a contributing virulence factor in the pathogenesis of some bacteria. Three out of 5000 clones screened from a genomic DNA library of *B. pseudomallei* were found to express protease activity. The clones were found to have the same sequence. The nucleotide sequence revealed an open reading frame (designated as metalloprotease A, *mprA*) encoding a 500-amino acid protein, MprA, with an estimated molecular mass of 50 241 Da. The predicted amino acid sequence shares homology with the subtilisin family of serine proteases. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Burkholderia pseudomallei*; Melioidosis; Protease

1. Introduction

*Burkholderia pseudomallei*, a Gram-negative bacterium, causes a glanders-like disease, melioidosis, in human and animals. The bacterium is a soil organism found mainly in Southeast Asia and Northern Australia. In Singapore, 22 to 90 cases of melioidosis were reported annually from 1990 to 1997 by the Quarantine and Epidemiology Department, Ministry of the Environment [1].

*B. pseudomallei* secretes a number of proteins that may play important roles in the pathogenesis of melioidosis. These secreted proteins have been found to have cytotoxic and proteolytic activities [2]. Ninety-one out of 100 isolates were found to be positive for lecithinase, lipase and protease [3]. Twenty environmental and clinical isolates were examined for exoproducts, some with protease, lipase, lecithinase and siderophore activities (prt/lip/let/sid) were found to be highly cytotoxic to HeLa cells, while the strains that expressed protease and siderophore activities (prt/sid) were found to have low to moderate cytotoxic activity to HeLa cells [4]. When the lethality of these strains were examined in Syrian golden hamsters, the animals inoculated with the strains (prt/lip/let/sid) displayed no signs of morbidity and mortality while the strains (prt/sid) killed all the hamsters. The roles played by various secreted exoproducts in the virulence of *B. pseudomallei* are not clear. Percheron et al. [5] have studied an exoprotease in *B. pseudomallei* that requires Zn$^{2+}$ for optimal production. Sexton et al. [6] has purified and characterized a protease with a molecular mass of 36 000 from *B. pseudomallei*. They have shown that a *B. pseudomallei* strain deficient in protease production was less virulent than the parental strain in an animal model of lung infection using diabetes-induced Sprague-Dawley rats. In another study, Gauthier et al. [7] showed that there is no correlation between virulence and level of protease activity when *B. pseudomallei* was injected into SWISS mice intraperitoneally.

Many extracellular bacterial proteases have been implicated to play important roles in virulence. To facilitate genetic analysis of the protease in order to determine its role as a virulence factor in melioidosis, we report the cloning and sequencing of the gene *mprA* encoding a protease expressed by *B. pseudomallei*. The *mprA* gene was found to encode for a serine metalloprotease that shared homology with the subtilisin family of proteases.
2. Materials and methods

2.1. Bacterial strains and culture conditions

*B. pseudomallei* strains ATCC 15682 (monkey isolate), ATCC 23343 (human isolate), human clinical isolates 9, 22, 59 and Jumari, pig isolates P6 and 21/96, and soil isolates 77/96, 78/96, 79/96 and 1009/96 obtained from E.H. Yap (Department of Microbiology, National University of Singapore) were streaked out from frozen bacterial stocks on LB agar plate (Difco Laboratories, Detroit, MI, USA) and incubated at 37°C for 2 days. Bacterial strains *B. mallei* ATCC 23344, *B. mallei* ATCC 10399 and *B. cepacia* ATCC 25416 were also obtained from E.H. Yap.

2.2. Construction of a genomic library

Chromosomal DNA of *B. pseudomallei* extracted using the Qiagen genomic DNA kit (Qiagen, Hilden, Germany) was partially digested with the restriction enzyme, Sau3AI and fractionated on agarose gel. DNA fragments of 2–4 kb isolated using a Qiagen gel extraction kit were ligated into *Bam* HI-digested Bluescript SK+ phagemid (Stratagene, La Jolla, CA, USA) with T4 DNA ligase (Gibco BRL, Life Technologies, Rockville, MD, USA) into Epi-curian Coli XL1-Blue supercompetent cells (Stratagene, La Jolla, CA, USA).

2.3. Detection of protease activity on agar plates and inhibition studies

Protease activity was detected by the dialyzed brain heart infusion (D-BHI) milk medium (Becton Dickinson and Co., Cockeysville, MD, USA) in agar plates [8]. The library was plated on the D-BHI milk agar plates and incubated at 37°C for 2 days and protease activity was detected as zones of hydrolysis around the colonies. Supernatants from 2-day-old culture grown in BHI media were collected for the inhibition studies. The protease activity was measured quantitatively by the enzymatic release of azo dye from azocasein (Sigma, St. Louis, MO, USA) by reading the optical density at 420 nm [9].

2.4. Southern blot analysis

A digoxigenin (DIG)-labeled polymerase chain reaction (PCR) product specific to the protease gene was amplified with the primers, 14F3 (5’ CCGCGCTCAGGATTCTGTC 3’) and 14R3 (5’ CGCGTTGCAGGGCTAAG 3’) (PCR DIG Probe Synthesis Kit, Roche Diagnostics, Mannheim, Germany) and used as probe. About 1.5 µg of genomic DNA from each bacterial isolate was completely digested with *Sau* I and *Eco* RI, ran on a 0.7% agarose gel and transferred onto a positively charged nylon membrane (Amersham Life Science, Buckinghamshire, UK). The membrane was hybridized with about 10 ng ml⁻¹ of the DIG-labeled PCR probe at 42°C overnight in DIG Easy Hyb solution (Roche Diagnostics, Mannheim, Germany). Denatured salmon sperm DNA (Stratagene, La Jolla, CA, USA) at 50 µg ml⁻¹ was added to the hybridization solution to increase the stringency.

2.5. Sequence analysis

Both strands of the cloned DNA were sequenced using the ABI 373 automated sequencer (PE Applied Biosystems, Fosters City, CA, USA). Nucleotide and amino acid sequences were analyzed with programs from DNASTAR (DNASTAR, Madison, WI, USA) and GenBank (National Centre for Biotechnology Information). The sequence data for the *mprA* gene has been submitted to the GenBank database under accession number AF254803.

2.6. Polyclonal antibody production

A peptide with 19 amino acid residues, VVPNDTRYSEQWGYFNPTA, corresponding to amino acid residues 157–175 of the predicted protein sequence, was synthesized (Bioprocessing Technology Centre, National University of Singapore) using the multiple antigen peptide system. The peptide in Freund’s complete adjuvant was used to immunize two female adult rabbits. The rabbits were boosted twice before the sera were collected 10 weeks after the first injection. The rabbits used were hybrids of Singapore local species with New Zealand white albino.

2.7. Western blot analysis

Cell lysates and supernatants from the clones and *B. pseudomallei* strains grown in BHI media for 2 days were prepared and separated on a 10% sodium dodecyl sulfate–polyacrylamide gel as described by Laemmli [10]. Proteins from the gel were transferred on to nitrocellulose. The membrane was first blocked with phosphate-buffered saline (PBS)–Tween (0.5%) containing 5% skim milk for 1 h, washed three times with PBS–Tween, then incubated with rabbit antiserum at 1:100 dilution and the enhanced chemiluminescence kit (Amersham Life Science, Buckinghamshire, UK) was used to visualize the proteins bound to the rabbit antibodies.

3. Results

3.1. Cloning and isolation of the *mprA* gene

Three protease positive clones, p1.1, p2.1 and p3.1, showing zones of clearance around the colonies were found by screening 5000 colonies of a genomic library on skim milk agar plates. The clones p1.1, p2.1 and p3.1, contained DNA inserts of 10.6-, 3.5- and 2.8-kb
DNA from *B. pseudomallei*, respectively. All three clones were found to contain the same overlapping sequence when aligned with an open reading frame (ORF) encoding for a protein of 500 amino acid residues (not shown).

The *mprA* gene was detected in a 4-kb fragment in all nine isolates of *B. pseudomallei* when the genomic bacterial DNA digested by *SacI* and *EcoRI* were analyzed by Southern blot (Fig. 1). The bacterial DNA from three human isolates, four soil isolates, one pig isolate and the reference strain, ATCC 23343, were used in the Southern blot. The *mprA* gene was not present in other closely related bacteria as shown in one *Burkholderia cepacia* and two *Burkholderia mallei* isolates analyzed.

Ordered sets of clones from exonuclease III deletion in the 5′ end of the insert were selected and their proteolytic activities were determined on skim milk agar plates. Attempts were made to introduce deletions in the 3′ end, but all the clones isolated did not express any protease activities indicating that the termination site was probably located very close to the 3′ end of the insert. Deletion analysis suggested that the expression of an active extracellular protease required approximately 1.9 kb of the inserted DNA (data not shown).

### 3.2. Nucleotide sequence of *mprA* gene

An ORF with an ATG initiation site located 7 bp downstream of the 5′-GGAG-3′ sequence that might function as a Shine–Dalgarno (SD) sequence [11] was found in the 1.9-kb insert. The G+C content of the insert is 70% compared to 68% for the whole genome [12]. The predicted amino acid sequence of the protease had a putative signal sequence containing 30 amino acids [13,14] with a potential signal peptidase cleavage site between Thr30 and Ala31 (Fig. 2). The mature protein has a predicted molecular mass of 47 250 Da and a predicted isoelectric point of 6.91.

### 3.3. Comparison of *MprA* with other proteases

The predicted amino acid sequence was found to share extensive homology to the subtilisin family of proteases. Fig. 2 shows that similarities of 42, 36, 35 and 33% were found when compared with proteases in the subtilisin family. The deduced amino acid sequence of *MprA* revealed that the catalytic residues of aspartic acid (Asp206), histidine (His264) and serine (Ser437) are conserved, as is the oxyanion-hole residue asparagine (Asn367) of the subtilisin family of protease [15].

### 3.4. Western blot analysis of *MprA*

The rabbit polyclonal antibody against a 19-amino acid peptide in the predicted ORF detected a protein of approximately 50 kDa (Fig. 3). Similarly, the protein was detected in the supernatant of the monkey isolate ATCC strain 15682, pig isolate P9 and clinical isolate 59. The supernatants of these isolates were shown to produce protease by the azocasein assay. The antibody barely detected a protein at about 50 kDa in the supernatant from the ATCC strain 23343, a human isolate which showed no activity using the azocasein assay, probably due to the low amount of protease expressed. The isolates appeared to express varying amounts of protease when cultured under similar conditions.

### 3.5. Effect of protease inhibitors on enzyme activity

The metal chelator, EDTA, inhibited protease activity but not the zinc chelator, 1,10 phenanthroline. The serine protease inhibitor, phenylmethylsulfonyl fluoride, also inhibited the protease activity. The protease was resistant to

**Table 1**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Activity (%)</th>
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<tr>
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<tr>
<td>EDTA</td>
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<td>90.6</td>
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<tr>
<td>Phenylmethylsulfonyl fluoride</td>
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<td>13.1</td>
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<tr>
<td></td>
<td>1 mM</td>
<td>16.2</td>
</tr>
<tr>
<td>1,10-Phenanthroline</td>
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<td>89.3</td>
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<td></td>
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<td>Aprotinin</td>
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<tr>
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<td>91.7</td>
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<tr>
<td>Pepstatin</td>
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<td>90.0</td>
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<tr>
<td></td>
<td>10 μM</td>
<td>90.6</td>
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Supernatants were obtained from protease clone p3.1 grown for 2 days in BHI with 50 μg ml⁻¹ ampicillin. 100 μl of the supernatant were incubated with 10 μl of metal-chelator or protease inhibitor or water as control for 30 min at 37°C. Then the protease activity was measured by the azocasein assay [9]. The results were calculated as [(OD₄₄₀ of treated supernatant)/(OD₄₄₀ of untreated supernatant)]×100. The averages of the protease activity from at least two experiments were shown.
Fig. 2. Comparison of amino acid sequence of MprA with other subtilisin-like proteases. The amino acid sequences of the protease of *B. pseudomallei* (BPMPRA), the extracellular protease of *Xanthomonas campestris* pv. *campestris* (XCPROA) (GenBank accession P23314 [17]), the *Bacillus* *lentus* subtilisin BL (BLSUBB) (GenBank accession P29599 [18]), with the M-protease of *Bacillus* sp. (BSPRTM) (GenBank accession PO99405 [19]) and with *Dichelobacter nodosus* extracellular basic protease precursor (DNBPRV) (GenBank accession P42779 [20]) were aligned. Identical amino acid residues are shown as dots and to maximize homology, gaps (dashes) were introduced into the sequences. Asterisks mark the conserved active-site residues, aspartic acid, histidine and serine residues of the subtilisin-like proteases. An open circle marks the oxyanion-hole residue asparagine. An arrow marks the potential signal peptidase cleavage site. The underlined amino acid sequence was used to generate rabbit polyclonal antibodies.

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cysteine and aspartate protease inhibitors such as aprotinin and pepstatin A. The reducing agent, dithiothreitol, has a slight inhibitory effect on the protease activity. The results summarized in Table 1, indicate that the protease is most likely a serine metalloprotease. The protease was stable at 60°C but the activity decreased at temperatures above 60°C.

4. Discussion

We have cloned and sequenced a novel protease gene, mprA from B. pseudomallei, most likely a serine metalloprotease, containing a signal sequence that could be cleaved to give a 47-kDa mature protein. Clone 18.1 containing the mprA gene inserted in the reverse orientation expressed protease indicating that the cloned DNA fragment contained it’s own promoter that works in Escherichia coli (data not shown).

Sixty-eight B. pseudomallei isolates from various sources were analyzed for the presence of the protease gene by PCR amplification. A fragment of appropriate size was amplified in all the isolates and the direct sequencing of the PCR product showed that the nucleotide sequence corresponded to those in the mprA gene (data not shown). Southern blot analysis of bacterial genomic DNA showed that the mprA gene was present in a 4-kb fragment in nine isolates of B. pseudomallei but not present in two B. mallei strains and one B. cepacia strain examined (Fig. 1). The mprA gene was found to be present in single copy in the B. pseudomallei isolates. The different intensities of the fragments for the different isolates seen in the Southern blot in Fig. 1 were due to loading of varying amounts of the digested DNA as indicated by the ethidium bromide stained gel (not shown).

Sexton et al. have purified and characterized a 36-kDa extracellular protease from B. pseudomallei that may be important in the pathogenesis of pneumonia [6]. Their protease was a metalloenzyme requiring iron for maximal activity. The 19 amino acids in the N-terminal, VVPNDTRYSEQWGYFNPTA, sequenced by Sexton et al. [6] for the purified protease were also present in the ORF of our predicted mprA gene (Fig. 2). In the present study, antibodies against this peptide of 19 amino acids detected a protein of about 50 kDa from the clones and B. pseudomallei isolates. The 36-kDa protease was not detected using this antibody. It is not clear whether the 36-kDa protease was a truncated version of the 50-kDa MprA or two different proteases were expressed. However, all the three clones obtained from the genomic library have the same ORF indicating that the MprA isolated may be an important protease in B. pseudomallei.

From our results on the protease expression shown in Fig. 3, ATCC 23343, a human isolate of B. pseudomallei, appeared to express a low amount of protease. Gaultier et al. have also found that the strain ATCC 23343 was a low protease producer and was avirulent in SWISS mice injected with the bacteria intraperitoneally [7]. Their results with other strains suggested that there was no correlation between virulence and level of protease activity when B. pseudomallei is injected in mice through the intraperitoneal route. This result differed from the observations by...
Sexton et al. that a protease-deficient strain of *B. pseudomallei* is less virulent than the parental strain in a rat model of lung infection [6]. Since different animal models and bacterial strains were used in the two studies, the results were far from conclusive on the role played by protease in pathogenesis.

De Shazer et al. showed that in the hamster infection model, the mutant that could not secrete protease, lipase and phospholipase C, possibly through a mutation in the common secretion pathway for these exoproducts, the lethal doses (LD₅₀) were 13 compared to < 5 for the wild-type isolate [16]. This suggests that exoproducts secreted by type II pathway probably played a minor role in *B. pseudomallei* pathogenesis. Their paper did not address the exact role played by the protease in virulence of *B. pseudomallei* as the protease is one of three proteins that was not secreted by the mutant.

Ninety-four percent of the *B. pseudomallei* isolates were found to produce extracellular protease [3], suggesting that it may have an important role in the virulence of *B. pseudomallei*. The cloning of a novel protease, MprA, from *B. pseudomallei* would allow further genetic manipulations to determine the role of protease in bacterial pathogenesis. The regulation of expression of the *mprA* gene can also be studied, since under similar culture conditions, different isolates were found to express different amounts of protease.

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**References**


